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(54) Title: ENZYME ASSAYS

(57) Abstract

Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino–acid corresponding to amino acid residue number 245 in *Photinus pyralis* which is such that the K_m for ATP of the luciferase is increased e.g. five–fold with respect to that of the corresponding non–mutated enzyme such that it is of the order of 500 μm – 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucloetides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real–time. Also disclosed are test–kits for *in vitro* assays.

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ENZYME ASSAYS

Technical Field

The present invention relates broadly to enzymes and methods suitable for assaying ATP. It further relates to specific applications for such assays.

Background Art

Intracellular ATP concentrations can vary 10-fold or more depending upon a cell's state of health or developmental stage. It is of great value to be able to measure fluctuations in intracellular ATP levels as a means of investigating e.g. the effects of drugs, toxins, hormones, environmental agents or disease on cells.

There is apparently at present no convenient method for analysing the concentration of ATP *in vivo*. For instance, in Dementieva et al (1996) Biochemistry (Moscow) Vol 61, No. 7., the intracellular concentration of ATP was measured in *E. coli* by calculating the total amount of ATP present using a recombinant luciferase, and dividing by an estimated total cell volume.

Such an indirect approach can at best produce only an estimate of the actual ATP concentration.

The measurement of ATP concentration in cells has also been performed using an *in vitro* coupled assay, such as that disclosed in the Sigma Diagnostic Kit Catalog No. 366, in which Phosphoglycerate kinase is used to convert 3-phosphoglycerate to 1,3 diphosphoglycerate in an [ATP]-dependent fashion. The 1,3 diphosphoglycerate is then converted to glyceraldehyde-3-P concommitantly with conversion of NADH to NAD, which can be monitored spectroscopically. The assay has a dynamic range up to 1 mM; the expected range is 380-620 μm when used with blood cells.

However it can be seen that, as with all coupled assays, the test is inevitably cumbersome to perform. Additionally it could not readily be adapted for *in vivo* use. It would thus be a contribution to the art to provide materials and methods which overcome some of the drawbacks of the prior art.

Disclosure of the Invention

In a first aspect of the invention there is provided a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme. Preferably the K_m is at least double that of the non-mutated enzyme, and more preferably at least around five, ten, or twenty times higher than that of the non-mutated enzyme.

Luciferases are, of course, already known in the art. In the presence of Mg^{2+}, luciferase (originally obtained from fireflies) catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2, pyrophosphate and light. This basic property (luciferin and ATP to produce light) is hereinafter referred to as 'luciferase activity'.

The term 'luciferase' as used in relation to the invention is intended to embrace all luciferases, or recombinant enzymes derived from luciferases which have luciferase activity. This explicitly includes recombinant mutant luciferases which have deletions, additions or substitutions to their amino acid structure provided that they retain luciferase activity. Such luciferases will typically have considerable homology (e.g. up to 70, 80, 90, or 99%) with wild-type enzymes. However the crucial technical feature of the luciferases of the present invention which distinguishes them from those of the prior art is that they have a mutation which causes an increase in the K_m for ATP of the luciferase as compared with that measured for a corresponding enzyme which differs only in it that it lacks that same mutation.

This increase K_m may be measured by the person of ordinary skill in the art by conventional enzyme assays, as described in more detail in the Examples below.

It should be noted that in the prior art, luciferase has sometimes been used as a marker for gene expression (*in vivo*) where its production in a cell is linked to a particular genetic control element. Luciferin is added exogenously and intracellular ATP concentrations, under almost all conditions, will be such that the enzyme is saturated. Thus the switching on of gene expression is signalled by light that is emitted in a quantitative manner according to the amount of active luciferase that is generated.

However it should be stressed that in the previously known systems it is generally the concentration of luciferase which is measured; this concentration is then correlated with a different event e.g. the efficiency of a promoter. Indeed it has, on occasions, been an object of the prior art teaching on luciferases to reduce the K_m for ATP (see e.g. WO 96/22376) which ensures that changes in the ambient [ATP] does not interfere with the assay.

Similarly the assay disclosed by Dementieva et al (1996) discussed above requires that all of the ATP be efficiently converted to light so that the total ATP present can be calculated. This approach requires a low K_m luciferase so that the enzyme operates at near maximal velocity until all the ATP is hydrolysed.

By making available luciferases which have an increased K_m compared with those already known in the art, the present inventors have for the first time opened up the possibility of using these enzymes to measure steady state ATP concentrations over range which was previously unsuitable. This is because, generally speaking, the relationship between enzyme velocity (V, as measured by light intensity) and substrate concentration (of ATP, where luciferin is in excess) is as follows:

$V = V_m . [ATP] / K_m + [ATP]$

It can therefore be seen that only when the K_m is greater than (or of a similar order as) the ambient [ATP] will there be a degree of proportionality between changes in [ATP] and changes in light intensity. Where the K_m is much less than the ambient [ATP], any changes in [ATP] will not tangibly effect the measured light intensity. Clearly the more sensitive the light detection is, the smaller the measurable changes in 'V' can be, and the smaller the K_m can be with respect to the [ATP] range being assessed.

For certain applications, e.g. *in vivo* measurements, it may be advantageous to have a luciferase wherein the K_m is of the order of between 400 μm to 1.4 mM e.g. 500 μm, 600 μm, 1 mM etc. However, as can be appreciated from the discussion above, the main criterion is that the K_m is not much less than the expected [ATP] range to be assessed, and the phrase 'of the order of' should be construed accordingly.

A particular expected **[ATP]** range which is important for physiological assays of blood cells is between 300 μm and 1 mM, or more particularly 380 μm and 620 μm, (cf. Sigma Diagnostic Kit, Catalog No. 366 discussed above). For other mammalian cells such as hepatocytes, the [ATP] range is 2.5 mM - 6 mM (see Dementieva et al (1996) discussed above. Use of the recombinant luciferases of the present invention for continuous assays in these ranges is particularly envisaged.

The disclosure of the present application makes such high K_m luciferases available for the first time. The prior art disclosures reveal only luciferases having a K_m of between 60 μm and 150 μm, which would be saturated in these ranges.

It is also advantageous, as with all enzymes used in assays, that the mutant enzyme retains sufficient activity (i.e. a high maximum turnover number, giving a high V_m) such that practical concentrations of enzyme can give detectable results.

Preferably the activity for ATP of the mutant is at least 5-100% of that of the corresponding wild-type; however reduced-activity as a result of the high K_m mutation can, if necessary, be compensated for by using more enzyme or more sensitive detection if required.

In one embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

It should be noted that the sequences of a number of luciferases from different sources have already been published in the literature, see e.g. WO 95/25798 for *P pyralis*; EP 0 524 448 for *Luciola cruciata* and *Luciola lateralis*. Other known *luc* genes include *Luciola mingrelica*, and *Lampyris noctiluca* (see Newby et al (1996) Biochemical J 313: 761-767.)

Whether an amino-acid in a luciferase 'corresponds' to number 245 in *P pyralis* (which is His in the wild-type, non-mutated enzyme) can be established by the person of ordinary skill in the art without difficulty as follows: the sequence of the luciferase is established (either from the literature or by sequencing); the sequence is aligned with *P pyralis*, for instance using commercially available software (e.g. "Bestfit" from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) or manually such as to demonstrate maximal homology and align conserved amino acids; the amino acid corresponding to number 245 in *P pyralis* is identified. An example of this is shown below using *L cruciata* - the corresponding amino acid in that case is number 247.

Once identified a mutant can be prepared e.g. by site directed mutagenesis by methods commonly used in the art and exemplified below.

Preferably corresponding amino-acid is substituted for an uncharged amino acid, for instance nonpolar (e.g. Ala) or uncharged polar (e.g. Asn, or Gln):

CLASS	EXAMPLES OF AMINO ACID
Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic:	Asp, Glu
Basic:	Lys, Arg, His

It should be noted that WO 95/18853 (PROMEGA) lists a large number (over 80) of *Pyrophorus plagiophtalamus* mutants which are reported to have altered spectral properties. However the K_m for ATP of the mutants is not reported, nor indeed discussed at any point in the application.

In another embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 318 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme. Correspondence may be assessed as above; preferably the amino acid (Ser in the wild type) is substituted for a bulkier one (e.g. Thr).

In preferred forms the mutant luciferases of the present invention incorporate one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light. Some suitable mutations are already known to those skilled in the art; see e.g. WO 95/25798 and WO 96/22376 and EP 0 524 448 for thermostability improving mutations (e.g. at positions corresponding to 354 and 215 of *P pyralis*).

Preferably the mutation causing the increased K_m itself improves one or more of these properties, particularly thermostability. It should be noted that an enhanced stability at around 37°C is especially advantageous for enzymes which are to be employed *in vivo*.

In a further embodiment the luciferases may be in the form of fusion proteins or incorporate polypeptide extensions. This may improve the ease by which they can be produced, localised *in vivo* or extracted and purified.

In a second aspect of the invention there is disclosed a recombinant polynucleotide encoding a mutant luciferase of the present invention, as described above.

In a third aspect there are disclosed vectors comprising a polynucleotide of the second aspect. For instance vectors further comprising a replication element which permits replication of the vector in a suitable host cell and/or a promoter element which permits expression of said polynucleotide in a suitable host cell. The promoter may be a constitutive promoter. Optionally the promoter element may be tissue- or organ-specific.

In a fourth aspect there is disclosed a host cell containing, or transformed with, a vector of the third aspect.

Optionally the host cell of the fourth aspect may express one or more further luciferases which have a lower K_m for ATP than those of the present invention, and possibly emit light of a different wavelength, such as to extend the useful range of any assay, and/or allow the use of a ratiometric assay i.e. one in which the activity of the high K_m mutant is compared with that of a further luciferase. The further luciferases may be recombinant non-mutant luciferases or recombinant mutant luciferases having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme (see e.g. WO 96/22376 .

Coloured mutants are disclosed in WO 95/18853 and in Ohmiya et al (1996) FEBS Letters 384: 83-86.

In a fifth aspect there is disclosed a process for producing a luciferase of the present invention comprising culturing a host cell as described in the fourth aspect.

In a sixth aspect there is disclosed a single cell organism consisting of a host cell as described above, or a multicellular organ or organism comprising it. The use of e.g. transgenic higher animals in which the luciferases of the present invention are expressed could allow *in vivo* study of [ATP] in different types of cell or tissue as described in more detail below. In particular, as ATP is present in virtually all living cells, any type of cell into which luciferase could be cloned, from bacterial to plant or animal, could be studied through the measurement of ATP changes.

Thus in a seventh aspect of invention there is disclosed a method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as described above.

Preferably the method comprises the following steps(a) the luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

The measurement in step (b) may be compared with a control value such as minimise base-line errors.

The assay can be *in vitro* or *in vivo*.

More preferably the material itself is a cell, in to which the luciferase is introduced e.g. by transforming the cell with a vector as described above. Alternatively the luciferase may be introduced into the cell by direct injection.

Equally the material measured may be part of a synapse i.e. the ATP is neurotransmitter.

Generally the assay will be most useful for real-time analysis (on a time-scale of seconds e.g. using a CCD camera, photomultiplier or photodiode) of events initiated by particular stimuli (e.g. addition of an active agent to the material). In this case the assay can monitor changes in [ATP] concentration over a relatively short time-scale. Such measurement will not, therefore, be greatly affected by longer time-scale events, such as changes in the concentration of luciferase in the system. These changes can be correlated with cellular events e.g. tissue necrosis may be associated with falling [ATP], fatigue in muscle likewise. Such continuous assays have hitherto not been possible.

Other possible applications include measuring the effect of drug treatments on various tissues; toxins and uncoupling agents on oxidative phosphorylation; bacterial infection; metabolic processes and stress (e.g. obesity and exercise); studies of brain activity (e.g. memory function and mental disorders) etc.

If appropriate the [ATP] can be measured periodically (rather than constantly) using photographic film.

Essentially the monitoring can be done in ways analogous to those already used in the art for other applications e.g. for the photonic detection of bacterial pathogens in living hosts disclosed by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In that paper a Hamamatsu intensified CCD camera was used to visualise *Salmonella Typhimurium* expressing luciferase during infection of a mouse. Equally a system equivalent to PET (positron emission tomography - as used in brain scans) could be used to achieve precise localisation of luciferase-generated light to allow the metabolism of specific body regions to be ascertained.

Generally speaking it will be necessary to introduce luciferin into the system being studied. By 'luciferin' is meant any co-factor which has luciferin activity i.e. can be used in conjunction with luciferase to cause light to be emitted in the presence of ATP. The manner by which this is introduced in to the system will depend on the system itself. For instance where animal cells are being studied, luciferin may be introduced by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part. Similarly when the system being studied is one or more plant cells, the luciferin may simply be introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

In a final aspect of the invention there is disclosed a test kit comprising a luciferase discussed above and further comprising one or more of the following(a) a buffer or dry materials for preparing a buffer; (b) ATP standards; (c) luciferin; (d) Dithiothreitol (e) instructions for carrying out an ATP assay.

The invention will now be further described with reference to the following none-limiting Figures, Sequence Listings and Examples.

Figures

Fig 1. shows plasmid pPW601a as described in Example 1.

Fig 2. for mutant H245A, (a) shows the plot of V against [ATP] and (b) shows 1/V against 1/[ATP] as described in Example 1.

Fig 3. shows a sequence comparison of one region of *P pyralis* (Pp) and the corresponding region of *L cruciata* (Lc) as described in Example 2.

Fig 4 is a graph showing light emission versus ATP concentration for mutant H245N.

Fig 5 . shows the effect of the addition of nutrient broth to luciferase-expressing E. coli cells pre-charged with Luciferin as described in Example 6.

Fig. 6 shows Seq ID No. 1 which is the nucleotide sequence of the wild type *luc* gene from *P pyralis*.

Fig. 7 shows Seq ID Nos. 2-5 which are the primers used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4) and the equivalent wild-type sequence (Seq ID No 5).

Fig. 8 shows Seq ID No. 6 which is the amino acid sequence of a high K_m mutant H245Q of the present invention, wherein amino acid 245 has been changed to Gln.

Examples

EXAMPLE 1: PRODUCTION OF RECOMBINANT HIGH K_M MUTANT LUCIFERASE

Except where otherwise stated, the methods employed were as those used by White et al (1996) Biochemical Journal 319: 342-350, which is concerned with thermostable mutants.

STARTING MATERIALS: Mutants were generated by site directed mutagenesis of the plasmid pPW601a (Fig 1) comprising the luciferase gene, *luc*, from *P pyralis*. The wild type *luc* gene from *P pyralis* is shown at Seq ID No. 1. Plasmid pPW601a was created by cloning the *luc* gene *Bam*HI/*Sst*I fragment from pGEM-*luc* (available from Promega) into pDR540 (available from Pharmacia). The unique *Xho*I site in the polylinker of the plasmid was removed to simplify the following procedures.

SITE DIRECTED MUTAGENESIS: Three mutagenic oligonucleotides were used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4). The equivalent wild-type sequence is shown at Seq ID No. 5. The oligonucleotides also introduced a silent mutation which destroys a unique Xmn I site in the *luc* gene - this did not result in an amino acid

substitution but facilitated mutant selection. The mutagenesis was carried out in accordance with the kit instructions of kit supplied by Clontech laboratories Inc, Palo Alto, California USA.

The amino acid sequence of H245Q is shown in Seq. ID No. 6.

ISOLATION OF PLASMID DNA & TRANSFORMATION: this was carried out by the method of Brinboim & Doly (1979) Nucleic Acids Research 7: 1513.

CELL CULTURE AND EXTRACTION: *E. coli* JM109 transformants were grown to an OD_{600} = 1.0. Aliquots of cells expressing mutant luciferases from plasmid pPW601a, were subjected to lysis as described in the Promega technical bulletin and the lysed extracts were then stored on ice prior to assay.

ASSAY OF Km OF MUTANT LUCIFERASES: luciferase assays were performed at 21°C using 100 μl of assay buffer (20 mM Tricine pH 7.8 containing 2.0 mM $MgSO_4$, 0.1 mM EDTA, 33mM dithiotheitol, 470 μM D-luciferin and ATP in the concentration range 6.25 - 800 μM). Each assay contained 5-10 μl of crude cell extract.

The plots of V against [ATP] and 1/V against 1/[ATP] for mutant H245A are shown in Fig. 2. Such plots kan be used to determine the K_m.

The results of each mutation and the recombinant Wild Type are shown in Table 1:

Table 1

Luciferase	K_m MgATP (μM)
r Wild Type	66
H245A	442
H245N	623
H245Q	1340
A215L*	65

* A215L is a thermostable mutant in which amino acid 215 is substituted with lysine (see WO 96/22376 - SECRETARY OF STATE FOR DEFENCE).

ASSAY OF THERMOSTABILITY OF MUTANT LUCIFERASES: the thermostability of H245N & H245Q was also tested, as compared with mutant A215L and the wild-type. Lysed crude extracts of cells containing luciferase activity were incubated at 37°C for set time periods. The thermostability of the mutant H245A was found to be very similar to that of the recombinant wild-type. The results are shown in Table 2:

Table 2

Enzyme	Remaining activity %			
	0	2	4	8 minutes
r Wild Type	100	64.8	36.6	26.6
A215L	100	101	88	84
H245N	100	96	61	46
H245Q	100	103	78.6	51.5

PURIFICATION: luciferases, e.g. incorporating the H245Q mutation, may be purified as described in White et al (1996) [supra]. Briefly, the cell lysates are centrifuged at 30000 g for 30 mins and the supernatant is fractionated with ammonium sulphate (30-55%). This fraction is resuspended and desalted. The desalted material was passed through a hydroxyapatite column and eluted with 10-200 mM sodium phosphate containing dithiothreitol. The luciferase containing eluant is dialysed and applied to a Mono Q anion-exchange column. The enzyme can be eluted with 0 to 500 mM NaCl.

EXAMPLE 2: IDENTIFICATION OF CORRESPONDING HIGH K_M MUTANTS

Fig 3. shows a sequence comparison of one region of *P pyralis* and the corresponding region of *L cruciata* as describe din Example 2. In this case it can be seen that amino acid 245 corresponds to 247.

EXAMPLE 3: EXPRESSION OF MUTANT LUCIFERASE IN MAMMALS

This can be achieved by methods analogous to those disclosed by Liu et al (1997) Nature Biotechnology 15: 167-173. In this method cationic liposomes are used to deliver plasmid DNA containing luciferase a gene to mice.

EXAMPLE 4: AN *IN VIVO* ATP ASSAY IN MAMMALS

This can be carried out by methods analogous to those used by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In this method luciferase expression in *S typhimurium* in mice is monitored using a CCD camera.

EXAMPLE 5: A KIT FOR AN *IN VITRO* ATP ASSAY

This may be provided as follows: luciferase H245Q; buffer; or dry materials for preparing a buffer; ATP for standards; luciferin; and instructions for carrying out an ATP assay.

EXAMPLE 6 : Assay for determining cell behaviour

Using a luciferase assay as described in Example 1, a plot of the photon count versus the ATP concentration was prepared for the H245N mutant. The results are shown in Figure 4.

In order to demonstrate how the enzyme of the invention can be used in studying cellular behaviour, a sample of recombinant E.coli cells which expressed the H245N mutant luciferase were rendered dormant by exhaustion of nutrients. The cells were charged with luciferin by 10 minutes immersion in p.H. 5.0 citrate buffer containing 1mM luciferin. They were then centrifugally washed, resuspended in 1ml Nutrient Broth and the luminescence monitored. The results are shown in Figure 5.

Using the mutant luciferase of the invention, the revival and growth of functional cells could be monitored.

CLAIMS

1. A recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme.

2. A luciferase as claimed in claim 1 wherein the K_m is at least double that of the non-mutated enzyme.

3. A luciferase as claimed in claim 2 wherein the K_m is at least five times higher than that of the non-mutated enzyme.

4. A luciferase as claimed in claim 1 wherein the K_m is of the order of 500 μm.

5. A luciferase as claimed in claim 1 wherein the K_m is of the order of 1 mM.

6. A luciferase as claimed in any one of the preceding claims having a V_m for ATP which is at least 5-100% of that of the corresponding wild-type.

7. A luciferase as claimed in any one of the preceding claims wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

8. A luciferase as claimed in claim 7 wherein the amino-acid has been substituted for an uncharged amino acid.

9. A luciferase as claimed in claim 8 wherein the amino-acid has been substituted for Ala, Asn, or Gln.

10. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Photinus pyralis* and wherein amino acid residue number 245 has been substituted.

11. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Luciola cruciata* and wherein amino acid residue number 247 has been substituted.

12. A luciferase as claimed in any one of the preceding claims incorporating one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light.

13. A fusion protein comprising a luciferase as claimed in any one of the preceding claims.

14. A recombinant polynucleotide encoding a luciferase as claimed in any one of the claims 1 to 12.

15. A replication vector comprising a polynucleotide as claimed in claim 14 further comprising a replication element which permits replication of the vector in a suitable host cell.

16. An expression vector comprising a polynucleotide as claimed in claim 14 further comprising a promoter element which permits expression of said polynucleotide in a suitable host cell.

17. A vector as claimed in claim 16 wherein the promoter element is tissue or organ specific.

18. A host cell containing a vector as claimed in any one of claims 15 to 17.

19. A host cell transformed with a vector as claimed in any one of claims 15 to 17.

20. A host cell as claimed in claim 19 which also expresses a second luciferase having a lower K_m for ATP.

21. A host cell as claimed in claim 20 wherein the second luciferase is selected from: (a) a recombinant non-mutant

luciferase; (b)a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme.

22. A process for producing a luciferase comprising culturing a host cell as claimed in any one of claims 19 to 21.

23. A host organism consisting of or comprising a host cell as claimed in any one of claims 19 to 21.

24. Use of a recombinant luciferase as claimed in any one of claims 1 to 12 for assaying the amount of ATP in a material, wherein the concentration of the ATP is expected to be between 300 μm and 6 mM.

25. A method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as claimed in any one of claims 1 to 12.

26. A method as claimed in claim 25 wherein (a) the luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

27. A method as claimed in claim 26 wherein the measurement in step (b) is compared with a control value.

28. A method as claimed in claim 26 wherein the measurement in step (b) is made in real-time.

29. A method as claimed in any one of claims 25 to 28 wherein the material measured forms part of a synapse.

30. A method as claimed in any one of claims 25 to 28 wherein the material is a cell and the luciferase is introduced into the cell.

31. A method as claimed in claim 30 wherein the luciferase is introduced into the cell by transforming the cell with a vector as claimed in any one of claims 15 to 17.

32. A method as claimed in claim 30 or claim 31 wherein the luciferin is introduced into the cell by direct injection.

33. A method as claimed in claim 30 or claim 31 wherein the cell is an animal cell and the luciferin is introduced into the cell by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part.

34. A method as claimed in claim 30 or claim 31 wherein the cell is a plant cell and the luciferin is introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

35. A test kit comprising a luciferase as claimed in any one of claims 1 to 12 and further comprising one or more of the following(a) a buffer or dry materials for preparing a buffer; (b) two or more measured portions of ATP suitable for preparing standard solutions; (c) luciferin; (d) instructions for carrying out an ATP assay.

Fig.1.

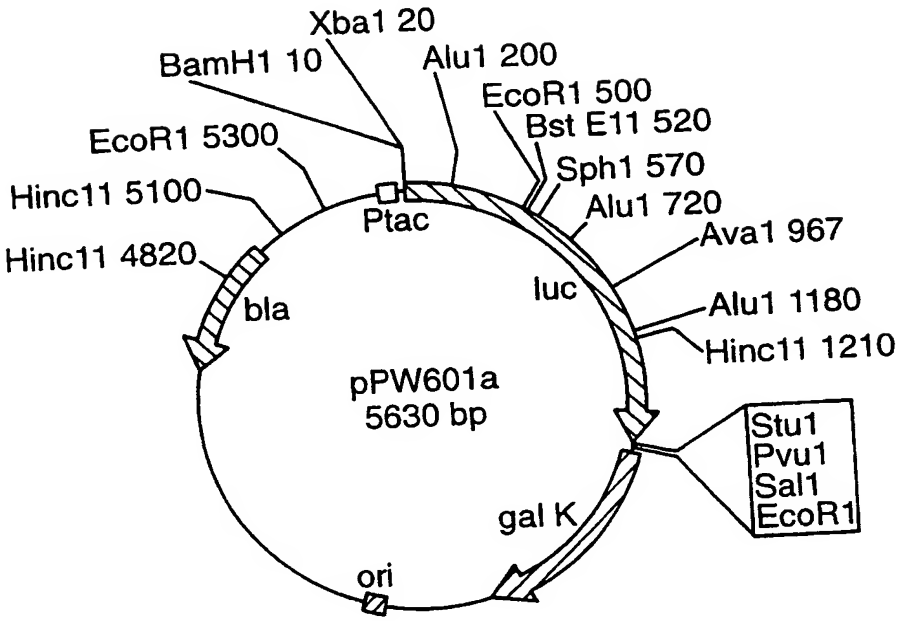
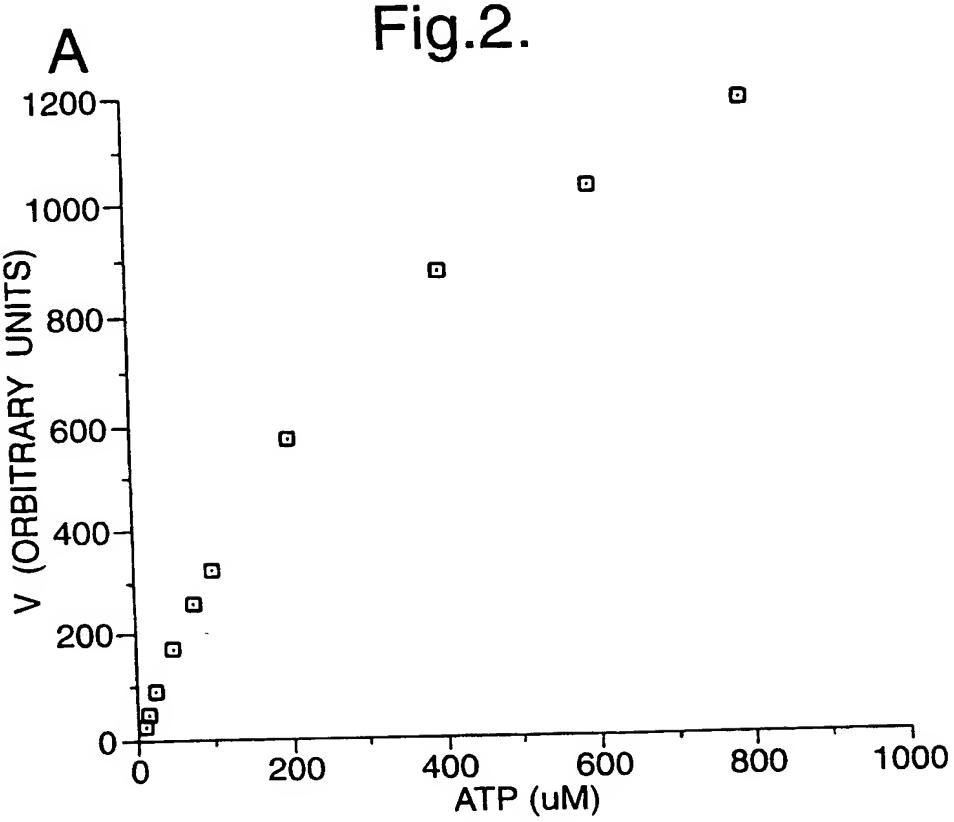


Fig.2.



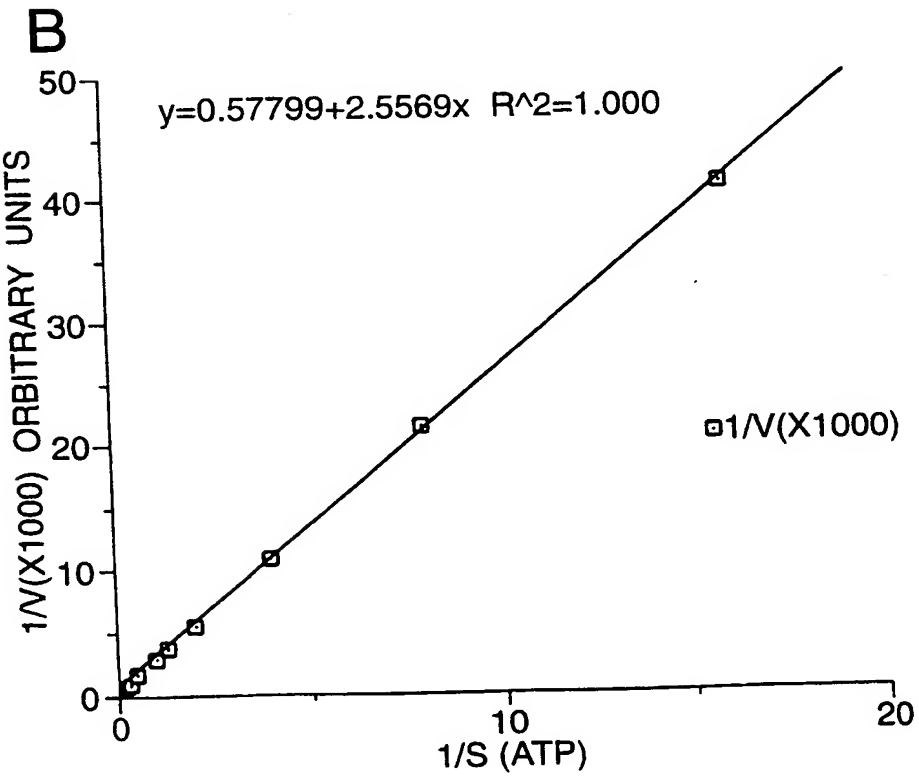


Fig.3.

```
                 H245
     230          ↓
      ↓
Pp   IIPDTAILSVVPFHHGFGMFTTLGYLIC        23/28 identical
Lc   VSPGTAVLTVVPFHHGFGMFTTLGYLIC
      ↑           ↑
     232         247

                     P318A
     301              ↓
      ↓
Pp   IDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPG        30/35 identical
Lc   LNKYDLSNLVEIASGGAPLSKEVGEAVARRFHLPG
      ↑               ↑
     303             320

                         S347T
     330                  ↓
      ↓
Pp   RFHLPGIRQGYGLTETTSAILITPEGDDKPGAVGKVVPFF        35/40 identical
Lc   RFNLPGVRQGYGLTETTSAIIITPEGDDKPGASGKVVPLF
      ↑                   ↑
     332                 349
```

Fig.4.

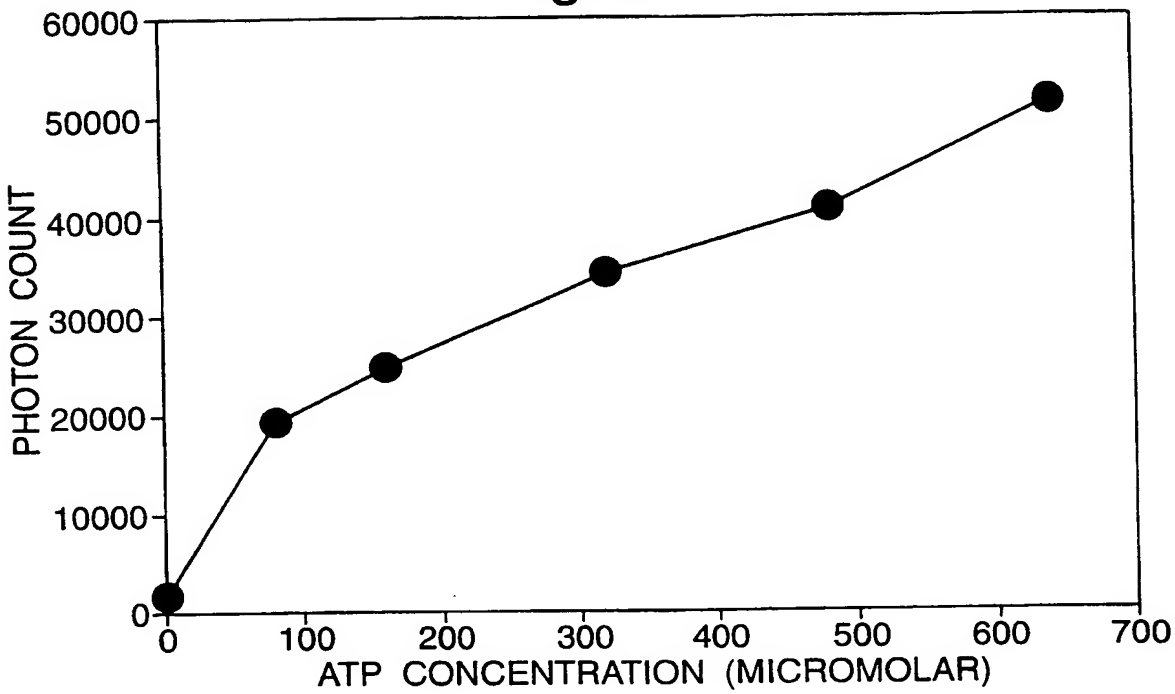


Fig.5.

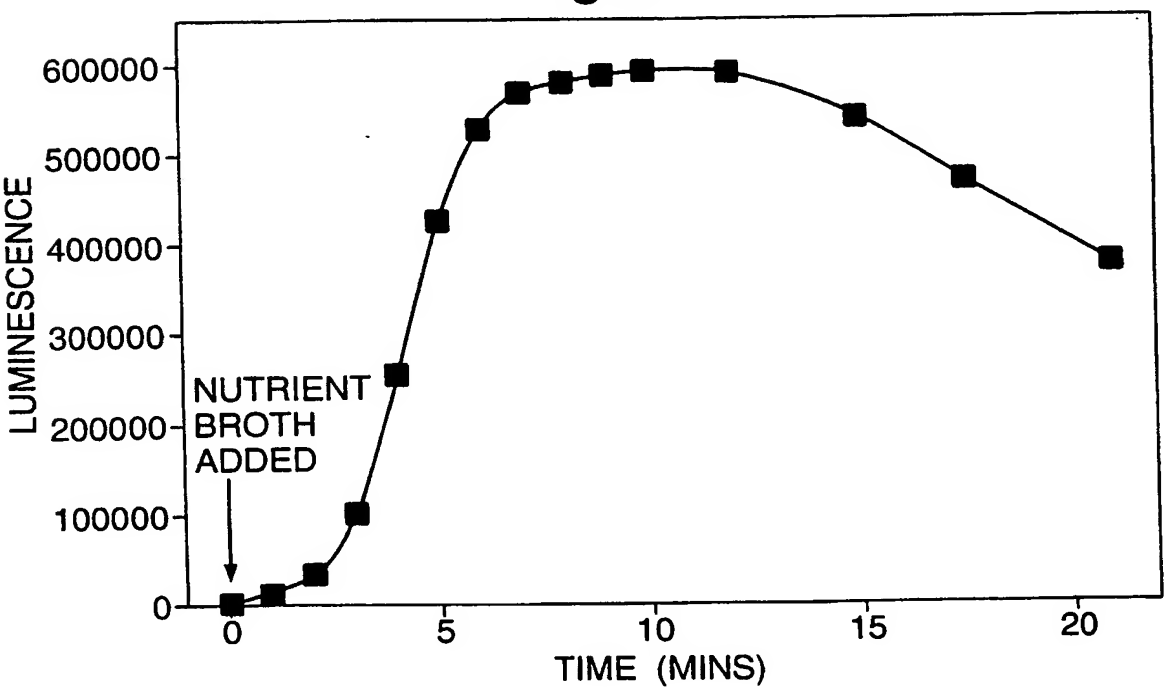


Fig.6.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1722 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 4..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAATGGAAG ACGCCAAAAA CATAAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT 60

GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA 120

ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG 180

TCCGTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC 240

GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA 300

GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC 360

ATTTCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG 420

CAAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG 480

GGATTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC 540

GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT 600

GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC 660

TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT 720

Fig.6 (Cont).

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA 780

TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT 840

TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT 900

CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT 960

TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA 1020

TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG 1080

GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG 1140

AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC 1200

GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT 1260

TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG 1320

TCTTTAATTA AATACAAAGG ATATCAGGTG GCCCCCGCTG AATTGGAATC GATATTGTTA 1380

CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA 1440

CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG 1500

GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG 1560

GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC 1620

ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC 1680

GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA 1722

Fig.7.

MUTAGENIC OLIGONUCLEOTIDES

Sequence ID No. 2

H245A:

736 737 744
↓↓ ↓
5' - GTT GTT CCA TTC CAT gcC GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 3

H245Q:

738 744
↓ ↓
5' - GTT GTT CCA TTC CAT CAg GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 4

H245N:

736 744
↓ ↓
5' - GTT GTT CCA TTC CAT aAC GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 5

WILD TYPE SEQUENCE:

721 H245 nt 753
↓ | | ↓
5' - GTT GTT CCA TTC CAT CAC GGT TTT GGA ATG TTT AC-3'

nt numbering is from luc gene sequence, CAA (nt 1) ....

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

Fig.8.

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35 40 45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Fig.8 (Cont).

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His Gln Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550

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(54) Title: ENZYME ASSAY FOR MUTANT FIREFLY LUCIFERASE

(57) Abstract

Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino–acid corresponding to amino acid residue number 245 in *Photinus pyralis* which is such that the K_m for ATP of the luciferase is increased e.g. five–fold with respect to that of the corresponding non–mutated enzyme such that it is of the order of 500 μm – 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucloetides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real–time. Also disclosed are test–kits for *in vitro* assays.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 98/01026

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/02 C12N15/53 C12N15/79 C12N15/82 C12N15/85
C12N15/63 C12Q1/66

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 6 C12N C12Q

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E.I. DEMENTIEVA ETAL.,: "Physicochemical properties of recombinant Luciola mingrelica luciferase andits mutant forms" BIOCHEMISTRY, vol. 61, no. 1, 1996, pages 115-119, XP002078631 Moscow, RU cited in the application see the whole document ---	1-3
A	WO 96 22376 A (SECR DEFENCE BRIT) 25 July 1996 cited in the application see abstract see page 1, paragraph 4-5 - page 2, paragraph 1 see page 3, paragraph 1; example 2 --- -/--	1,10-12, 20,24, 25,35

[X] Further documents are listed in the continuation of box C.

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

Inter ɔnal Application No
PCT/GB 98/01026

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Biochemistry (Moscow), Vol. 61, No. 1, 1996

Physicochemical Properties of Recombinant *Luciola mingrelica* Luciferase and Its Mutant Forms

E. I. Dementieva,[1,2] E. E. Zheleznova,[1] G. D. Kutuzova,[1] I. A. Lundovskikh,[1] and N. N. Ugarova[1]

Submitted September 6, 1995; revision submitted October 12, 1995.

Physicochemical properties of recombinant *L. mingrelica* luciferase synthesized by *E. coli* cells were studied. The catalytic and spectral properties of the recombinant luciferase were similar to those of the native one, but the former was less stable due to the presence of an additional Cys residue. Mutant forms of *L. mingrelica* firefly luciferase with point mutations Cys-82→Ala, Cys-260→Ala, Cys-393→Ala, and Thr-204→Asp were constructed using the method of site-specific mutagenesis. Cys-82,260,393→Ala mutations changed slightly the K_m for ATP and luciferin but did not influence k_{cat}. The Cys-393→Ala mutant appeared to be more stable than the native luciferase. Mutation Thr-204→Asp resulted in a 8-fold increase in the ATP binding constant and a 2-fold increase in k_{cat}, indicating that Thr-204 may be located in the ATP-binding region of the luciferase. Dithiothreitol, ethylene glycol, bovine serum albumin, and trehalose had a stabilizing effect on the native, recombinant, and mutant luciferases.

KEY WORDS: firefly luciferase, mutagenesis, recombinant luciferase, luciferase stability.

Luciferases isolated from different species of fireflies differ somewhat in amino acid sequences and physicochemical characteristics although they catalyze the same reaction of luciferin oxidation by air oxygen in the presence of ATP [1]. The genes of firefly luciferases of *Photinus pyralis* [2, 3], *Luciola cruciata* [4], *Luciola lateralis* [5], *Luciola mingrelica* [6, 7], *Pyrocoelia miyako*, *Hotaria parvula* [8] and of four beetle luciferases of *Pyrophorus plagiophtalamus* [9, 10] have been cloned so far.

The mechanism of luciferin oxidation [11] and the kinetic scheme of the reaction catalyzed by firefly luciferase [12] have been already established; nevertheless, the structure of the luciferase active site and the groups of the protein which take part in catalysis have not yet been determined. In order to obtain information about amino acid residues which influence the bioluminescence spectrum and luciferase stability, mutant forms of luciferases have been obtained. Using random mutagenesis followed by isolation of clones which express enzymes different from the native one, five mutant forms producing bioluminescence with color from green (λ_{max} = 558 nm) to red (λ_{max} = 612 nm) have been obtained for *Luciola cruciata* luciferase [13]. According to sequence data, the change in bioluminescence spectrum results from single amino acid substitutions. For example, the single mutation His-433→Tyr led to a red shift of the bioluminescence maximum by 50 nm.

Wood obtained hybrid mutant forms of luciferases of the luminous click-beetle *P. plagiophtalamus* by cutting the luciferase cDNAs and joining the parts of different genes [10]. Analysis of clones which produce different colors of bioluminescence allowed him to reveal the amino acid groups responsible for strong shifts of bioluminescent spectra. For example, the difference between yellow-green and yellow bioluminescence results from the substitutions Arg-223→Glu and Leu-238→Val.

Computer analysis of amino acid sequences has established a high degree of correlation between position of bioluminescence maximum and physicochemical properties for the 223-247 sequence of different luciferases, including their mutant and hybrid forms [14]. This suggests that this amino acid sequence creates the microenvironment for the excited product (oxyluciferin), which determines the bioluminescence spectrum.

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Point mutations of the residue in position 217 strongly effect the thermal stability of *L. cruciata* [15] and *L. lateralis* [16] luciferases. Mutants where residue 217 was changed to hydrophobic amino acids (Thr-217→Ile, Val for *L. cruciata* luciferase; Ala-217→Ile, Leu, Val for *L. lateralis* luciferase) appeared to be the most stable.

Previous studies have shown that cysteine residues of firefly luciferase may be involved both in catalysis and in protein stabilization. Firefly luciferase is very sensitive to N-ethylmaleimide and other SH-group modifying reagents. Modification of SH-groups resulted in a dramatic decrease in catalytic activity and change in bioluminescence color from yellow-green to red [17, 18].

Thermal inactivation of luciferase may involve unfolding of the protein and oxidation of SH-groups [18]. Luciferases differing in the number of cysteine residues have different stability. The luciferases of *Luciola* species (*L. cruciata, L. mingrelica*) contain 7-8 cysteine residues, and they are less stable than *P. pyralis* luciferase which has only 4 cysteine residues [6, 7]. To determine the role of SH-groups in catalysis and protein stabilization, we chose three cysteine residues conservative for luciferases from different firefly species and changed them to alanine using site-specific mutagenesis to obtain proteins with point mutations Cys-82→Ala, Cys-260→Ala, and Cys-393→Ala.

Comparison of amino acid sequences of luciferases with those of ATP-utilizing enzymes (4-coumarate:CoA ligase, 2,3-dihydroxybenzoate:ATP ligase, gramicidin S synthetase) revealed homologous regions which are probably responsible for the interaction of the luciferases with ATP (AMP) [6, 7]. These are regions 200-210 and 410-460. We proposed that the appearance of negative charge on the proposed ATP-binding region will influence the catalytic properties of luciferase and constructed the protein with point mutation Thr-204→Asp.

The present work is devoted to the study of physicochemical properties of the recombinant *Luciola mingrelica* firefly luciferase and its mutant forms with point substitutions Cys-82,260,393→Ala and Thr-204→Asp in comparison with the properties of the native enzyme.

MATERIALS AND METHODS

Homogeneous *Luciola mingrelica* firefly luciferase was prepared by the previously described procedure [19]. The previously constructed plasmids pJGR and pGK2 were used for expression of the recombinant *L. mingrelica* luciferase in *E. coli* cells. pGK2 plasmid, used for expression of mutant luciferases, is a modified pBluescript plasmid carrying the insert of luciferase cDNA [6, 7]. *E. coli* cells, strain LE 392, were transformed with the corresponding plasmid and used for preparation of large amounts of luciferases. *E. coli* cells were grown in LB medium containing ampicillin (100 μg/ml) and were broken with lysozyme. Luciferase was isolated from cell lysate, purified to homogeneity using the previously described procedure [6, 7], and stored at −70°C.

We used Sephadex G-25, DEAE-Sepharose, and Blue Sepharose (Pharmacia, Sweden) as chromatographic carriers. Bacto-tryptone and yeast extract were from Difco (USA), ATP from Reanal (Hungary), DNA sequencing kit from USB (USA), and [^{32}P]dATP from Amersham (UK). Acids and salts were of "specially purified" grade or were twice crystallized. All solution were prepared using Milli-Q purified water.

Mutant forms of the luciferase gene were obtained in collaboration with T. Baldwin (Texas A&M University, College-Station, Texas, USA). Twenty one base pair oligonucleotides corresponding to the chosen regions of the luciferase cDNA with one or two nucleotide mismatches (GC instead of TG for Cys→Ala mutation) at the central position were synthesized. Mutagenesis was performed as described by Kunkel [20] using single-stranded uracil-containing DNA. All mutations were confirmed by DNA sequencing of the resulting mutant cDNAs using plasmid form of Bluescript by the Sanger method with [^{32}P]dATP.

Luciferase activity was assayed by measuring the maximum intensity of light emitted during the enzyme reaction at saturating concentrations of substrates. The reaction mixture containing 0.05 M Tris-acetate buffer, pH 7.8, 2 mM EDTA, 10 mM $MgSO_4$ (0.4 ml), a solution of one of the substrates (4 mM ATP or 1 mM luciferin) (0.3 ml), and luciferase solution (10 μl) were put into a polystyrene cell 10 mm in diameter. Then a solution of the second substrate (0.3 ml) was quickly added and the bioluminescence intensity was recorded on a LKB 1250 luminometer (Sweden). Luciferase activity was expressed in arbitrary units: 1 arb. unit = 1 mV = $1 \cdot 10^9$ quanta/sec.

Protein concentration was determined spectrophotometrically at 280 nm (1 mg/ml of the protein corresponds to 0.75 optical density units) and using the Bradford method [21]. Fluorescence and bioluminescence spectra were recorded on a LS-50B fluorimeter (Perkin Elmer, UK). Binding constants of luciferase with luciferin ($K_{s\,LH_2}$) were determined by the fluorescent titration method. The addition of luciferin resulted in quenching of tryptophan fluorescence of the protein and a binding constant was calculated using Stern–Volmer equation for the static type of quenching [22]:

$$\frac{F_0}{F} = 1 + \frac{[LH_2]}{K_{s\,LH_2}}, \qquad (1)$$

TABLE 1. Physicochemical Characteristics of the Native, Recombinant, and Mutant Luciferases of *L. mingrelica* Fireflies

Enzyme	Specific activity × 10^9, arb. unit per mg	$K_{m\,ATP}$, mM	$K_{m\,LH_2}$, μM	$K_{s\,LH_2}$[a], μM	k_{cat}, sec^{-1}
Native luciferase	1-1.5	0.15 ± 0.03	20 ± 4	80 ± 8	0.010 ± 0.002
Recombinant luciferase	1.1	0.16 ± 0.03	24 ± 5	110 ± 10	0.010 ± 0.003
Cys-82→Ala mutant	0.9-1.0	0.60 ± 0.20	16 ± 3	200 ± 30	0.008 ± 0.002
Cys-260→Ala mutant	1	0.80 ± 0.10	67 ± 11	200 ± 30	0.012 ± 0.003
Cys-393→Ala mutant	1	0.58 ± 0.08	36 ± 7	130 ± 30	0.011 ± 0.002
Thr-204→Asp mutant	1	1.20 ± 0.30	97 ± 12	100 ± 10	0.021 ± 0.005

[a]Determined using fluorescence titration method by quenching of luciferase fluorescence with luciferin.

where F_0 is fluorescence intensity of the protein in the absence of the quencher (luciferin), F is fluorescence intensity of the protein in the presence of luciferin (fluorescence of protein not bound with luciferin), and $[LH_2]$ is the concentration of the quencher (luciferin).

The plot of relative fluorescence (F_0/F) versus concentration of the quencher (luciferin) was a straight line, the slope of which gives the value of $K_{s\,LH_2}$.

RESULTS AND DISCUSSION

Preparation of Recombinant and Mutant *Luciola mingrelica* Luciferases. Recombinant luciferase synthesized by *E. coli* cells carrying the pJGR plasmid with the *L. mingrelica* luciferase gene (without mutant changes) is a fusion protein containing 11 additional amino acid residues of *V. fisheri* bacterial luciferase at the N-terminus: Met-Asn-Lys-Cys-Ile-Pro-Met-Ile-Ala-Ser-Lys-Met (firefly luciferase)-... . The genes of *L. mingrelica* firefly luciferases with point mutations Cys-82→Ala, Cys-260→Ala, Cys-393→Ala, and Thr-204→Asp were inserted into the pGK2 plasmid; therefore, unlike the recombinant luciferase synthesized on the pJGR plasmid, the mutant luciferases did not contain the additional amino acid residues at their N-termini.

E. coli cells carrying the pJGR plasmid were able to produce ~50 mg of active luciferase per liter of cell culture, i.e., 20 times higher than cells containing the pBluescript plasmid with the luciferase gene and several times higher than the cells carrying the plasmids with trc- and temperature-sensitive λPr-promoters. For cells carrying the pGK2 plasmid the yield of luciferase was 5 mg per liter of cell culture. The yield of homogeneous enzyme (after purification of cell lysate by chromatographic methods) was 10-15%.

Catalytic Properties of Recombinant and Mutant Luciferases. The physicochemical and catalytic characteristics of the native, recombinant, and mutant *L. mingrelica* luciferases are presented in Table 1. Cys-82→Ala, Cys-260→Ala, Cys-393→Ala, and Thr-204→Asp mutations had no effect on the specific activity and bioluminescence and fluorescence spectra of luciferase. The specific activity of all recombinant luciferases appeared to be equal to that of the native luciferase. The bioluminescence spectra were similar for all luciferases studied; the maximum intensity was observed at 570 nm.

The maximal intensity of tryptophan fluorescence in proteins may lie within the region from 330 to ~350 nm depending on the polarity of the environment of the tryptophan residue, increasing polarity shifting the fluorescence maximum towards longer wavelengths [22]. For all the luciferases studied, the fluorescence maximum was observed at 340 nm (λ_{ex} = 290 nm), this indicating a polar environment of the single tryptophan residue in the *L. mingrelica* luciferase. The absorption spectra of the luciferases also remained unchanged. These data suggest that the mutations did not change the conformation of the protein around Trp-419.

Table 1 also presents the kinetic characteristics of the bioluminescence reaction catalyzed by the native, recombinant, and mutant luciferases. The Michaelis constants (K_m) calculated from the dependences of maximum bioluminescence intensity on concentration of one of the substrates, the concentration of the second substrate being constant and saturating, are complex combinations of rate constants of different steps of luciferin oxidation. The catalytic constant (k_{cat}) characterizes the rate-limiting step of the enzyme reaction, decomposition of the enzyme–oxyluciferin complex [12]. $K_{s\,LH_2}$, determined using the fluorescence titration method, characterizes binding of luciferase with its substrate, luciferin.

The values of $K_{m\,ATP}$ and $K_{m\,LH_2}$ for the native and recombinant (pJGR plasmid) luciferases coincided. $K_{m\,ATP}$ increased ~4-5-fold for the cysteine mutants, and ~8-fold for the threonine mutant. For Cys-260→Ala and Thr-204→Asp mutants, $K_{m\,LH_2}$ increased only slightly. The binding constant of the protein with luciferin ($K_{s\,LH_2}$) increased ~1.5-2-fold for the recombinant and mutant luciferases.

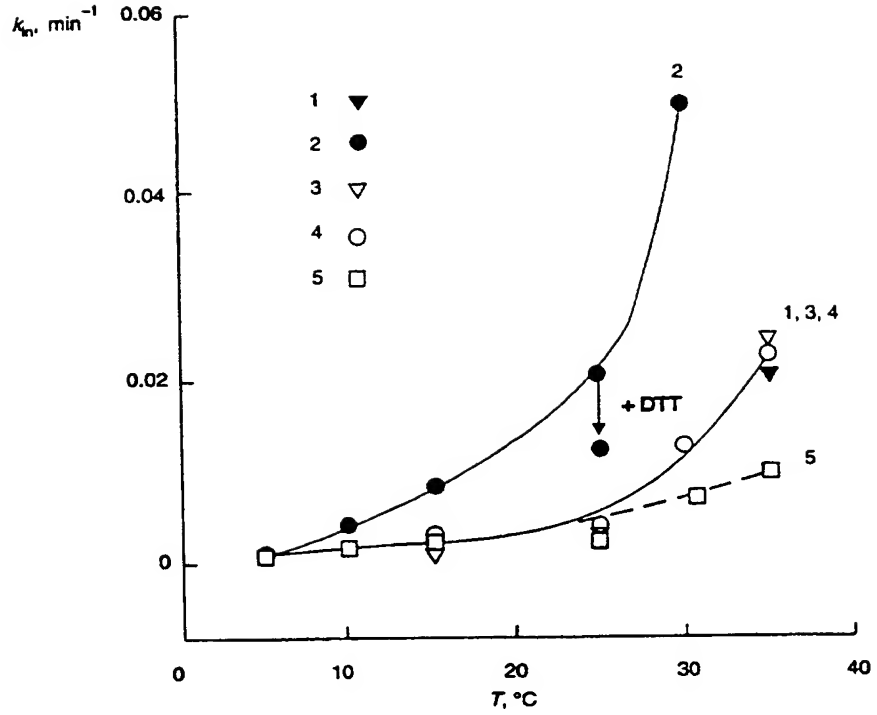

Fig. 1. Inactivation constants of native, recombinant, and mutant luciferases at 5-35°C (inactivation conditions were the same as in Table 2): 1) native luciferase; 2) recombinant luciferase (PJGR plasmid); 3) Cys-82→Ala mutant; 4) Cys-260→Ala mutant; 5) Cys-393→Ala mutant.

The values of k_{cat} appeared to be practically the same for all the luciferases studied except for the Thr-204→Asp mutant, for which a twofold increase in k_{cat} was observed. Thus, mutations of Cys-82, 260, and 393 residues had only a slight effect on binding of luciferase with substrates and this effect may be due to conformational changes in the protein molecule caused by the mutations.

Thus, we concluded that amino acid residues Cys-82, Cys-260, and Cys-393 of the *L. mingrelica* luciferase are not directly involved in the catalysis. For the Thr-204→Asp mutant, decrease in the ability to bind ATP and, simultaneously, increase in the constant of the rate-limiting step of the reaction (dissociation of the enzyme–product complex) were observed. These data confirm that Thr-204 is located in the neighborhood of the luciferase ATP-binding site. The appearance of negative charge instead of neutral weakened binding of the negatively charged ATP molecule. At the same time, binding of one of the reaction products (AMP) was also weakened, this resulting in the increase in the constant of the rate-limiting step.

Stability of the Recombinant and Mutant Luciferases. The luciferases with Cys→Ala point mutation had one SH-group less that the native enzyme. The recombinant luciferase synthesized on pJGR plasmid had an additic cysteine residue in the N-terminus. We studied the in ence of temperature and enzyme concentration on ir tivation constants (k_{in}) of the native, recombinant, mutant luciferases and the influence of substrates different additives on the stability of these proteins.

For all studied *L. mingrelica* luciferases, k_{in} at 2 did not depend on protein concentration within interval 10^{-9}-10^{-6} M. Cys-82→Ala and Cys-260→ mutations did not effect the luciferase stability in temperature interval 5-35°C (Fig. 1); hence, Cys-82 260 residues are not responsible for protein inactivat and are probably located inside the protein glot beyond the reach of oxidants. For the Cys-393→ mutant, the inactivation constants at 5-35°C were times lower than that of the native luciferase.

The decrease in stability of the recombinant lucife may result from the change in protein conformation to the presence of 11 additional amino acid residue the N-terminus and also with the existence of a cyst residue among them. A change in luciferase conforma should effect the absorption, fluorescence, and biolu nescence spectra and kinetic parameters of the lucife

TABLE 2. Influence of Additives on the Stability of Native, Recombinant, and Mutant Luciferases at 25°C[a]

Enzyme	Inactivation constant × 10^3, min^{-1}						
	no additives	luciferin (0.3 mM)	ATP (2 mM)	dithio-threitol (1 mg/ml)	ethylene-glycol (10%)	albumin (1 mg/ml)	trehalose (300 mg/ml)
Native luciferase	2.7 ± 0.08	3.0 ± 1.0	2.8 ± 1.0	2.7 ± 0.5	0.30 ± 0.05	0.43 ± 0.05	1.4 ± 0.1
Recombinant luciferase	20 ± 1	20 ± 1	21 ± 1	12 ± 1	6.0 ± 0.5	1.6 ± 0.1	12.9 ± 0.5
Cys-82→Ala mutant	3.0 ± 0.7	3.0 ± 1.0	2.8 ± 0.8	3.0 ± 0.8	0.30 ± 0.05	–	–
Cys-260→Ala mutant	4.2 ± 0.6	4.1 ± 1.8	4.3 ± 1.2	4.5 ± 1.0	1.8 ± 0.7	–	–
Cys-393→Ala mutant	1.9 ± 0.7	2.0 ± 0.4	1.8 ± 1.1	1.5 ± 0.5	0.30 ± 0.06	–	–

[a]Inactivation conditions: 0.05 M Tris-acetate buffer, pH 7.8, 2 mM EDTA, 10 mM $MgSO_4$, $5 \cdot 10^{-8}$ M luciferase.

catalyzed reaction. However, the spectra of the recombinant luciferase did not change in comparison with the native one and the kinetic parameters also remained practically unchanged. Addition of dithiothreitol, which is able to stabilize proteins by protecting SH-groups from oxidation, decreased the k_{in} of the recombinant luciferase but did not effect the stability of all the other luciferases (Table 2). From these data we may conclude that the most probable reason for the decrease in stability of the recombinant luciferase in comparison with the native one is the existence of the additional cysteine residue in the N-terminus of the fusion protein, rather than a change in the protein conformation.

Addition of the substrates, luciferin and ATP, did not change the stability of either native or mutant enzymes. The stability of all the luciferases increased 2-9-fold in the presence of ethylene glycol (10%), which increases the viscosity of the solution and thus prevents unfolding of the protein globule. Addition of other proteins, e.g., bovine serum albumin, also had a stabilizing effect on the recombinant and native luciferases (Table 2).

A stabilizing effect of trehalose (100 mg/ml) on the *P. pyralis* luciferase has been described in the literature [23]. We also observed an increase in stability of recombinant and native luciferases (~2 times at 25°C) in the presence of trehalose. It should be noted that concentrations of trehalose of 200-300 mg/ml also caused a 2-fold increase in luciferase activity in comparison with the initial value.

Thus, our comparative study of physicochemical properties of native, recombinant, and mutant *L. mingrelica* luciferases showed that the amino acid residues Cys-82, Cys-260, and Cys-393 are not involved in catalysis, but the SH-group of Cys-393 destabilizes the luciferase. Thr-204 is probably located in the ATP-binding region of the luciferase. The recombinant luciferase synthesized with *E. coli* cells carrying the pJGR plasmid is less stable than the native luciferase due to the presence of an additional cysteine residue in the N-terminus. This destabilizing effect may be reduced by addition of dithiothreitol, albumin, or trehalose.

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Bioluminescent Click Beetles Revisited

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In studying beetle bioluminescence in the early 1960s, Dr McElroy and his colleagues found that the Jamaican click beetle, *Pyrophorus plagiophthalamus*, was capable of emitting different colours of light. They further found that the luciferin substrate used by this beetle was the same as that in the firefly, demonstrating that the different colours of bioluminescence were due to differences in the structure of the luciferases. We have recently cloned cDNAs from this beetle species which code for at least four different luciferases. The luciferases are distinguishable by their different colours of bioluminescence when expressed in *Escherichia coli*. The sequence differences between these different luciferases are few, so the amino acids responsible for the different colours of emission must also be few. Through the construction of hybrid luciferases, by rearranging fragments of the original cDNA clones, we have identified some of these amino acid determinants of colour.

Keywords: Firefly luciferase; click beetle luciferases; bioluminescence spectra

INTRODUCTION

In 1963, a scientific expedition to Jamaica was led by William McElroy to study bioluminescence. There they encountered the 'kitty boo', a local name for the large bioluminescent click beetle, *Pyrophorus plagiophthalamus*. The beetle attracted the scientists' attention because of its ability to emit different colours of bioluminescence, a feature not found among species of true fireflies (Seliger *et al.*, 1964). It has two sets of light organs, a pair on the dorsal surface of the head, and a single light organ in a cleft on the ventral surface of the abdomen. The dorsal organs emit greenish light, while the ventral organ usually emits yellow or orange light. But the differences in colour do not occur only within individual specimens. Considerable variation also occurs between specimens for each set of organs. The colour emitted from the dorsal organs can range from green to yellow-green, and that of the ventral can range from green to orange. It was shown by these scientists that the differences in colour were not due to differences in the substrates of the luminescent reactions. The luciferases of these click beetle, or any other bioluminescent beetle, utilise ATP and the same luciferin that was first elucidated in the chemistry of the firefly luciferase. It was concluded that the different colours of bioluminescence were caused by subtle differences in the interaction of the substrates with the enzyme. Unfortunately, attempts to study these luciferases further were limited by the difficulties of collecting sufficient quantities of the beetles. Recently, we have been able to overcome this problem with the use of

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molecular cloning techniques. cDNAs generated from the ventral light organ of this beetle have been cloned and expressed in *Escherichia coli* (Wood *et al.*, 1988a, 1988b). These cDNA clones are of four different types, distinguishable by the colour of light emitted by the luciferases they code: green (546nm), yellow-green (560nm), yellow (578nm), and orange (593nm). These cDNA clones not only accord a practical source of the click beetle luciferases, but also provided a means of separating the different type since each clone is expressed in a separate host. Given here is the first report on the structural features of these click beetle luciferases that are responsible for the colour of light emitted.

AMINO ACID SEQUENCES AND THE DETERMINANTS OF COLOUR

Four cDNA clones, each capable of eliciting one of the four colours of bioluminescence in *E. coli*, were sequenced. With this information the amino acid sequences of the corresponding luciferases were deduced (Fig. 1). The open reading frames of each cDNA have a coding potential for 543 amino acids, seven amino acids less than that of the firefly luciferase cDNA. From a lysate of *E. coli* expressing click beetle luciferase, Western blot analysis revealed the presence of a single antigenic band which comigrates with the native enzyme (Wood *et al.*, 1988b). Since it is unlikely that a post-transitional cleavage of the luciferase could occur at a common site in both beetles and bacteria, such cleavages probably do not occur in either host. However, the resolution of the blots is not sufficient to rule out deletions of less than about twenty amino acids. Other possible post-translational modifications to the luciferases are also unlikely to occur in either host for similar reasons. In the least, such modifications are limited to those that would not result in a significant alteration in the migration of the luciferases in SDS-polyacrylamide gel electrophoresis. The simplest explanation of the Western blot data is that the luciferases are expressed in either host directly as the mature enzymes from their mRNAs.

Analogous observations have been made previously in the comparison of native firefly luciferase with that produced from its cDNA in *E. coli*. In this case, it was also shown that firefly luciferase produced by *in vitro* translation of its mRNA also comigrates with native enzyme (Wood *et al.*, 1984), this further indicating the absence of substantial post-translational modifications. However, some form of modification at the N-terminus of the native enzyme is implicated by our inability to obtain an amino acid sequence by Edman degradation. It is not at present known whether the N-terminus of firefly luciferase expressed in *E. coli* is also modified. Nevertheless, for both the firefly and click beetle luciferases, it is certain that there are no modifications required for enzymatic activity that are unique to the beetle metabolism, since the luciferases are active when expressed in exogenous hosts such as *E. coli*. Similarly, for the click beetle luciferases, variation in colour also is independent of possible beetle-specific modifications. Given the general lack of similarity between beetle and *E. coli* metabolisms, it is unlikely that there are any essential modifications of any sort. Thus, we work from the premise that the effective structure of the luciferases is simply the folded polypeptide chains which are coded by the cDNA clones.

Comparison of the amino acid sequences of the four luciferases shows a high degree of similarity. Pairwise comparisons of the sequences reveal from 94% to 99% identity (Fig. 2). This is in contrast to their comparison with the firefly luciferase sequence, with which they are less than 50% identical (Wood *et al.*, 1988b). In terms of amino acids, the sequences differ from each other by 5 to 32 residues. This information can be used to construct an evolutionary tree of the luciferases (Fig. 3) (Fitch and Margoliash, 1967), which shows that the luciferases have evolved in the order of the colour they emit. The earliest of the click beetle luciferases is the green-emitting luciferase, and the latest is the orange-emitting luciferase. The amino acid differences between the click beetle luciferases are the only differences between the *E. coli* capable of expressing the different colours of bioluminescence. Therefore, this set of amino acid differences must be responsible for the differences in the colour of bioluminescence.

The effect that these amino acid differences have on the spectra of bioluminescence is very uniform. This can be demonstrated in a plot of the peak positions versus their respective widths at half maximal intensity for each of the four colours (Fig. 4). These parameters are presented in terms of wave numbers since this parameter is

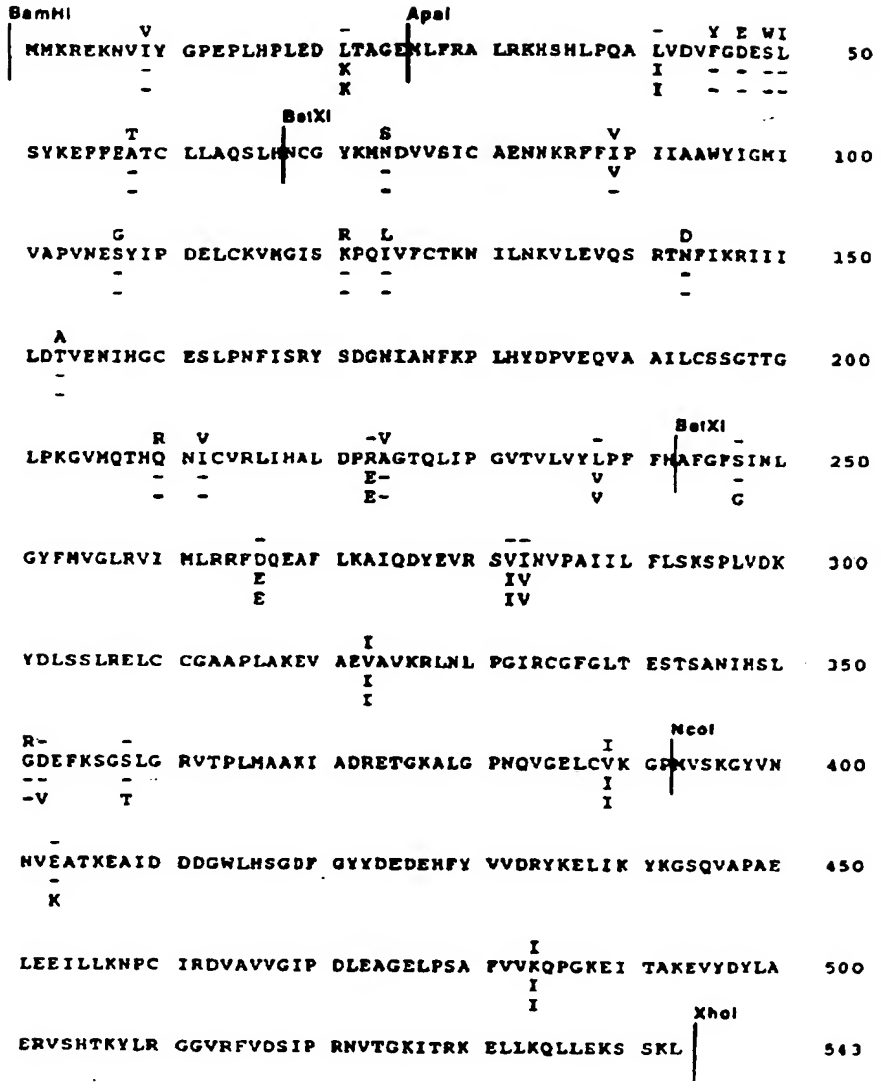

Figure 1. Amino acid sequences of the four click beetle luciferases capable of emitting four different colours. Shown in entirety is the sequence of the yellow-green-emitting luciferase. Above and below that sequence are shown only the amino acids at positions where the other luciferase sequences are different. Shown above the sequence are the amino acid differences for the green-emitting luciferase. Shown one line below the sequence are the amino acid differences for the yellow-emitting luciferase, and shown two lines below are the amino acid differences for the orange-emitting luciferase. A dash indicates no difference from the yellow-green emitting luciferase. Shown in bold type are the relative positions of restriction endonuclease site in the corresponding cDNA clones (the first BstXI site is not found in the green-emitting clone). The number on the right indicate the positions of the last amino acid in each line.

green	100%	96%	95%	94%
yellow-green	20	100%	97%	97%
yellow	23	11	100%	99%
orange	28	14	5	100%
	green	yellow-green	yellow	orange

Figure 2. Pairwise comparisons of the sequences of the four click beetle luciferases. The percentage identities of the sequences are shown in the upper right; the number of amino acid differences between the sequences are shown in the lower left (shaded area)

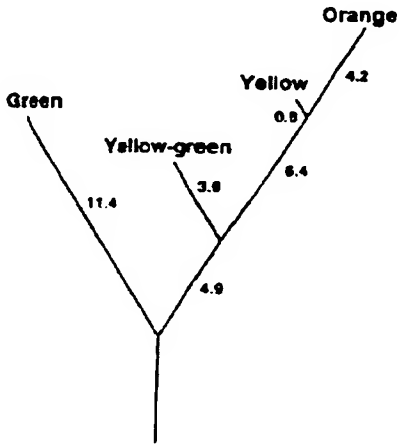

Figure 3. Evolutionary tree of the click beetle luciferases. Distances on the tree branches are given in number of amino acid differences between the sequences. The root of the tree was determined by comparison with the firefly luciferase

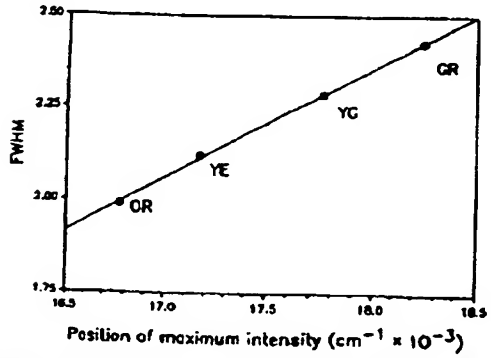

Figure 4. Plot of peak position versus peak width for each of the click beetle luciferases. Peak position is given as the wave number at which maximal intensity is found. Peak width is given as the width of the spectrum, in wavenumbers, found at half of the maximal intensity

proportional to the energy of the photons emitted. In this form, the peak positions and widths are descriptive of the energy states of the light-emitting complex. The emitting complex refers to both the specific oxyluciferin fluorophore and its associations with the protein. The uniform manner in which these parameters vary suggests that discrete changes in the nature of the light-emitting complex are not effectuated to produce the alterations in colour. Hypothetically, such discrete changes could be tautomerization or ionization of the emitter, or coupling of the emitter to other conjugated pi-bonded systems. Instead the predictable variation in the peak shapes is symptomatic of a modifying effect on the light emitter that can be varied in a continuous manner. Such modifying effects could be general changes in the dielectric constant around the emitter, or alterations of the electronic environment around specific key atoms in the emitter complex. The fact that the spectra of the four colours are nearly evenly spaced should not be construed to be a natural quantization of the system. Spectra taken from the ventral organ of live click beetles reveal other colours of bioluminescence, within the range of colours of the clones, which do not match any of the four colours from the clones (Biggley *et al.*, 1967). Presumably there are other members of this highly conserved group of click beetle luciferases that have not yet been cloned, which are capable of emitting different colours. Among all bioluminescent beetles there exists a large number of subtle shades in the colour of bioluminescence.

It should be remembered that the number of amino acid differences between the four sequences represents only an upper limit to the number required to produce the alteration in colour. As will be shown below, the number of amino acids that determine the colour of biolu-

minescence may be only a few. In the simplest *a priori* model, the colour would be determined by the chemical properties of four different amino acids found as a *single* position in all four luciferase sequences. Changes in the characteristics of the amino acids at this position, such as charge or hydrophobicity, would modify the colour of emission from one clone to the next in the uniform manner observed. However, with only the limited set of twenty amino acids available, it is difficult to find a uniform procession of chemical properties that could account for all four colours. The situation is worse when also included in the model are the other colours from the click beetle luciferases that have not yet been cloned. The fact is, comparison of the click beetle sequences reveals that there is no position in all four luciferases which changes to more than one other amino acid. Thus, as is typically the case, the role of individual amino acids to enzymatic activity is more subtle than the simplest model would predict.

METHOD FOR CONSTRUCTION OF HYBRID LUCIFERASES AND SPECTRAL ANALYSIS

Site-directed mutagenesis has become the standard method for determining the function of specific amino acids within an enzyme. However, to be practical, the method requires knowledge of which amino acids to modify. In our case, there are a total of 31 site which are variant between the four luciferase sequences. To mutagenize each one would be very time-consuming and costly. As initial experiments, to reduce the number of options, we chose a method that would allow us to change several amino acids simultaneously (Fig. 5). Since the luciferase sequences vary by only a small amount, most of the restriction endonuclease cleavage sites in the corresponding cDNA sequences are conserved in all four clones. We are therefore able to express hybrid luciferases in *E. coli* by cleaving the cDNA clones with restriction endonucleases, and recombining the DNA fragments in new arrangements. Often, though we are exchanging large regions of sequence information, we are only changing the identities of a few amino acids. By analysing the pattern of amino acid substitutions for a large number of such rearrangement hybrids, we hoped to identify a subset of key residues that affect the colour of bioluminescence. For this type of

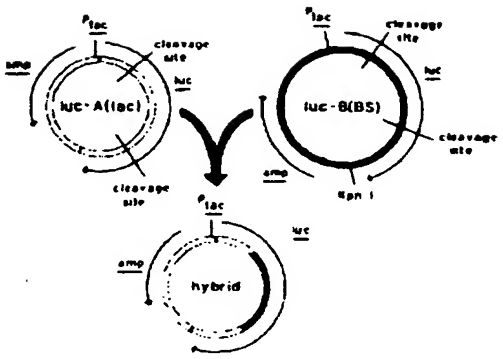

Figure 5. Method of constructing hybrid luciferases. Luc-A(tac) is the *tac*-containing vector (upper left). Luc-B(BS) is the Bluescript-based vector (upper right). Each vector contains a luciferase cDNA (*luc*) and a penicillin resistance gene (*amp*). The hybrid luciferase cDNA is constructed by cutting each parent vector at the 'cleavage site', and recombining the appropriate fragments.

analysis we assume that the variant amino acid position function independent of each other, and look for experimental evidence to the contrary. For the most part, this assumption has been consistent with our observations.

To allow verification afterwards that the rearrangement hybrids we were constructing were indeed made properly, we used a strategy involving two different types of plasmids. One plasmid is a small expression vector which contains the *tac* promoter. At present it is the vector which yields the greatest expression of bioluminescence. The other plasmid, Bluescript, was derived from the cloning vector with which the luciferase clones were originally isolated. This plasmid can be distinguished from the *tac*-containing vector by the presence of a unique **KpnI** restriction site. The construction is performed by cleaving both plasmids, each containing a different luciferase cDNA clone, into two fragments with a chosen set of restriction endonucleases. One fragment contains a portion of the luciferase cDNA and the entire plasmid vector. The other fragment is the remaining portion of the cDNA. A *tac* vector containing a hybrid cDNA is formed by ligating the vector-containing fragment from the parent *tac* vector, with its complementary fragment from the Bluescript vector. The incorporation into the final product of the correct vector-containing fragment, with its associated portion of the luciferase cDNA, can be quickly confirmed by the absence

of the KpnI site. The other fragment, which contains only luciferase sequence, must be confirmed by DNA sequencing since there are no unique restriction sites to identify one luciferase cDNA from another. The one exception to this is the green-emitting clone, which is lacking a BstXI site which is found in the other three cDNA clones.

The spectra of bioluminescence from *E. coli* containing click beetle luciferases can be measured either from intact cells or from cell lysates. It has been shown for each of the luciferases that the spectra measured by either method are identical (Wood *et al.*, 1988b). Since it is technically simpler and less time-consuming, we chose to measure our spectra directly from living cells. The cells are grown on nitrocellulose filters on top of nutrient agar. To initiate bioluminescence, the filters are removed from the agar and soaked with luciferin for 5 to 10 minutes as described (Wood and DeLuca, 1987). The filters are then blotted dry and placed in the spectrometer for measurement.

The spectrometer is a Fastie–Ebert type as described (Seliger *et al.*, 1964). The output of the photomultiplier tube is amplified and digitally converted for direct input into an IBM PC compatible computer. Each sample was scanned five times at a rate of approximately 8 nm/s over a total period of 5 minutes. Noise in the spectra was reduced by applying a digital curve-smoothing routine to the data. The set of five spectra was then corrected for the wavelength-dependence sensitivity of the photomultiplier tube, and for time-dependent variation in the intensity of the bioluminescent sample. The final spectra was then computed as the average of the five original spectra. The precision of this method varies, dependent on intensity of the sample. Except for very weak samples, there is a greater than 95% confidence that two spectra are different if their maxima differ by 2 nm or more. This confidence is greater for the more intense samples. For very weak samples, several sets of spectra may be averaged together to reduce the effects of noise.

IDENTIFICATION OF SPECIFIC AMINO ACIDS WHICH AFFECT THE COLOUR OF BIOLUMINESCENCE

By using different combinations of restriction enzymes on the cDNA clones, the four sequences were recombined to form four sets of rearrangement hybrids with five to eight members per set (Fig. 6). These hybrids, combined with the four original clones, form a total of 31 different luciferase sequences. Fig. 1 shows the relative positions of restriction sites which were used in the amino acid sequences of the luciferases. Also used in the constructions were two sites in the vectors near the ends of the cDNA inserts: BamHI at the 5′-end, and XhoI at the 3′-end. The first set of hybrids was constructed using NcoI and BamHI. This separates the last two variant amino acid positions from the remainder of the sequences. The second set of hybrids was constructed using ApaI and XhoI, which separates the first two variant positions from the remainder. The two BstXI sites, which separate approximately the central third of the variant positions, were used to construct the third set. This could not be done with the green-emitting clone because the first BstXI site was not present. This fact was used in the construction of the fourth set, which used the BstXI sites and the BamHI site to rearrange the final third of the variant position. For those clones which contained two BstXI sites, this resulted in cleavage of the fragment which contains only luciferase sequence into two. These fragments were combined and treated in the procedure as if they were only one, which was possible because the BstXI sites are not self-compatible. Thus, in the final ligation, the fragments can only recombine in one orientation.

To simplify nomenclature, we refer to the green-emitting clone as luc-GR, the yellow-green-emitting clone as luc-YG, the yellow-emitting clone as luc-YE, and the orange-emitting clone as luc-OR. The hybrids are referred to as 'luc-' followed by a number and a letter. The number refers to the set of hybrids which the clone is derived from, and the letter is an arbitrary distinction between the members of the set. Thus luc-2d is a hybrid that came from the second set of hybrids.. A group of amino acids substitutions are referred to as a set of changes, and are indicated as shown in this example: [R_{223},L_{238}→E,V] indicates that arginine at position 223 changes to glutamate, and that leucine at position 238 changes to valine. The inverse set would be changes in the opposite direction, i.e. [G_{223},V_{238}→R,L]. The amino acid suubstitutions required to change one luciferase into another are indicated as shown: luc-GR→2b [$V_{?}$,$L_{2?}$→I,K] indicates the necessary amino acid substitutions

Wild Type			
luc-GR	GR	VL.LYEWIT.SVGRLDARVRVL.SDVIIRDSI.EI	546 nm
luc-YG	YG	IL.LFDSLA.NISKINTQIRAL.SDVIVGDSV.EK	560 nm
luc-YE	YE	IK.IFDSLA.NVSKINTQIEAV.SEIVIGDSI.EI	578 nm
luc-OR	OR	IK.IFDSLA.NISKINTQIEAV.GEIVIGVTI.KI	593 nm
x NcoI, BamHI hybrids			
luc-1a	GR*YG	VL.LYEWIT.SVGRLDARVRVL.SDVIIRDSI*EK	546 nm
luc-1b	GR*OR	VL.LYEWIT.SVGRLDARVRVL.SDVIIRDSI*KI	546 nm
luc-1c	YG*GR	IL.LFDSLA.NISKINTQIRAL.SDVIVGDSV*EI	559 nm
luc-1d	YG*OR	IL.LFDSLA.NISKINTQIRAL.SDVIVGDSV*KI	561 nm
luc-1e	YE*YG	IK.IFDSLA.NVSKINTQIEAV.SEIVIGDSI*EK	579 nm
luc-1f	YE*OR	IK.IFDSLA.NVSKINTQIEAV.SEIVIGDSI*KI	578 nm
luc-1g	OR*GR	IK.IFDSLA.NISKINTQIEAV.GEIVIGVTI*EI	593 nm
luc-1h	OR*YG	IK.IFDSLA.NISKINTQIEAV.GEIVIGVTI*EK	593 nm
x ApaI, XhoI hybrids			
luc-2a	YG*GR	IL*LYEWIT.SVGRLDARVRVL.SDVIIRDSI.EI	546 nm
luc-2b	OR*GR	IK*LYEWIT.SVGRLDARVRVL.SDVIIRDSI.EI	546 nm
luc-2c	GR*YG	VL*LFDSLA.NISKINTQIRAL.SDVIVGDSV.EK	561 nm
luc-2d	OR*YG	IK*LFDSLA.NISKINTQIRAL.SDVIVGDSV.EK	560 nm
luc-2e	GR*YE	VL*IFDSLA.NVSKINTQIEAV.SEIVIGDSI.EI	579 nm*
luc-2f	YG*YE	IL*IFDSLA.NVSKINTQIEAV.SEIVIGDSI.EI	578 nm
luc-2g	GR*OR	VL*IFDSLA.NISKINTQIEAV.GEIVIGVTI.KI	593 nm*
luc-2h	YG*OR	IL*IFDSLA.NISKINTQIEAV.GEIVIGVTI.KI	593 nm
x BstXI hybrids			
luc-3c	OR*YG*OR	IK.IFDSLA*NISKINTQIRAL*GEIVIGVTI.KI	580 nm
luc-3d	YE*YG*YE	IK.IFDSLA*NISKINTQIRAL*SEIVIGDSI.EI	563 nm
luc-3e	YG*OR*YG	IL.LFDSLA*NISKINTQIEAV*SDVIVGDSV.EK	577 nm
luc-3f	YE*OR*YE	IK.IFDSLA*NISKINTQIEAV*SEIVIGDSI.EI	578 nm
luc-3g	YG*YE*YG	IL.LFDSLA*NVSKINTQIEAV*SDVIVGDSV.EK	577 nm
luc-3h	OR*YE*OR	IK.IFDSLA*NVSKINTQIEAV*GEIVIGVTI.KI	594 nm
x BstXI, BamHI hybrids			
luc-4a	GR*YG	VL.LYEWIT.SVGRLDARVRVL*SDVIVGDSV.EK	550 nm
luc-4b	GR*YE	VL.LYEWIT.SVGRLDARVRVL*SEIVIGDSI.EI	553 nm
luc-4c	GR*OR	VL.LYEWIT.SVGRLDARVRVL*GEIVIGVTI.KI	571 nm
luc-4d	YG*GR	IL.LFDSLA.NISKINTQIRAL*SDVIIRDSI.EI	559 nm
luc-4e	OR*GR	IK.IFDSLA.NISKINTQIEAV*SDVIIRDSI.EI	573 nm

Figure 6. Sequence of hybrid luciferases. Each set of hybrids is indicated in bold type by the restriction endonuclease sites used in its construction. Each line thereafter corresponds to a different hybrid within the set. The first entry of each line is the hybrid name. The second entry indicates the origin of the fragments used to construct the hybrid shown in their correct order. The third entry shows, in succession, the amino acids at every position in the four luciferase sequences where there is at least one difference between the sequences. The '.' in the series of amino acids indicate the positions of the restriction endonuclease sites. The restriction sites of actual use for the construction of a particular set of hybrids are indicated by '*'. The last entry of each line indicates the wavelength at which the intensity maxima occurs for the spectra of each hybrid luciferase. Maxima values marked with an asterisk are from spectra that were very weak, so that the exact value is somewhat uncertain.

required to change the green-emitting luciferase to hybrid 2b.

The results of the hybrid experiments can be best understood in terms of deviations from the native enzyme sequences for each luciferase. We will begin by examining luc-YG, whose spectra is most like that of the firefly luciferase. Changes to both the first two and the last two variant position did not produce any change in the spectrum of bioluminescence. These changes are luc-YG→2c [I_9→V], luc-YG→2d [L_{21}→K], luc-YG→1c [K_{484}→I], and luc-YG→1d [E_{403},K_{484}→K,I]. All of these changes produced luciferases whose spectral maxima were at 560 nm (within the limits experimental error). By combining these sets, we can form a new set, identified as luc-YG→YG', which describes changes in luc-YG that have little or no effect on its spectrum. This new set is luc-YG→YG' [I_9,L_{21},E_{403},K_{484}→V,K,K,I]. For three members of this set, the substituting amino acids have very different chemical properties. The change of E_{403} to K_{403} is one of the only two substitutions possible between any of the luciferases where the charge on the amino acid

reverses. In luc-YG→3e [R_{223},L_{238}→E,V] the spectrum shifts from 560 nm to 577 nm, which is indistinguishable from the spectrum of luc-YE. Luc-YG→3g [I_{89},R_{223},L_{238}→V,E,V] has the same effect. The difference between these sets, [I_{89}→V], therefore apparently has no effect on the spectrum and can be added to the set of substitutions which do not affect luc-YG, i.e. luc-YG→YG' [I_9,L_{21},I_{89},E_{403},K_{484}→V,K,V,K,I]. The difference between luc-YG→YE and luc-YG→3e is [L_{21},L_{41},I_{89},D_{226},$V_{282}I_{283}$,V_{323}, V_{389},K_{484}→K,I,V,E,IV,I,I,I]. By subtracting luc-YG→YG' from this, the set [L_{41},D_{226},V_{282} I_{283},V_{323},V_{389}→I,E,IV,I,I] is formed. It may be inferred indirectly that this set also has little effect on the spectra of luc-YG since the spectra of luc-YE and luc-3e are virtually identical. This can be tested directly by luc-YG→3d, which after removing the members of luc-YG→YG' forms this set inferred to have little effect on the spectrum. The spectral maxima of hybrid luc-3d is 563 nm, 3 nm higher than luc-YG. Thus, while this verifies that this set has little effect on the spectrum, it does have a measurable effect. This also demonstrates that it is both a necessary and sufficient condition that the amino acid substitutions which produce most of the spectral shift between luc-YG and luc-YE are in the set [R_{223},L_{238}→E,V].

As was found for the luc-YG, changes to the first two and last two variant positions of luc-YE have no effect on its spectrum. Combining luc-YE→1e, luc-YE→1f, luc-YE→2e, and luc-YE→2f, we get the set luc-YE→YE' [I_9,K_{21}, E_{403},I_{484}→V,L,K,K]. Luc-YE→3f [V_{89}→I] also produces no shift in the spectrum and so, as with luc-YG, it can be included in luc-YE→YE' to form [I_9,K_{21},V_{89},E_{403},I_{484}→V,L,I,K,K]. Since luc-YE→OR [V_{89},S_{247},D_{352},S_{358},E_{403}→I,G,V,T,K] with the members of luc-YE→YE' removed is [S_{247},D_{352},S_{358}→G,V,T], the amino acid substitutions that shift the spectra of luc-YE to luc-OR must be in this subset. By reasoning similar to that used to identified the set which can shift the spectra of luc-YG to luc-YE, the inverse of this set must contain the amino acid substitutions which can shift the spectra of luc-YE to nearly to luc-YG, i.e. [E_{223},V_{238}→R,L] shifts the spectra of luc-YE to 563 nm.

The spectral shifts caused by [S_{247},D_{352},S_{358}→G,V,T] and [R_{223},L_{238}→E,V] can be shown to act largely independently of each other by examining luc-YG→OR [L_{21},L_{41}, R_{223},L_{238},S_{247},D_{266},$V_{282}I_{283}$,V_{323},D_{352},S_{358},V_{389}, E_{403},K_{484}→K,I,E,V,G,E,IV,I,V,T,I,K,I]. After removing the members of luc-YG→YG', this set can be divided into three subsets: [R_{223},L_{238}→E,V], [S_{247},D_{352},S_{358}→G,V,T], and [L_{41},D_{266},$V_{282}I_{283}$,V_{323},V_{389}→I,E,IV,I,I]. The first set is the set of substitutions shown to be largely responsible for the spectral shift of luc-YG to luc-YE, and the second set are those responsible for the spectral shift of luc-YE to luc-OR. The third set is the set shown to have a small but measurable effects on the spectra of luc-YG. Therefore, the spectral shift from luc-YG to luc-OR, 33 nm, appears to rely on both of the first two sets of effective substitutions. It can be shown that these two sets act independently by applying each to luc-YG. The effect of [R_{223},L_{238}→E,V] is already described by luc-YG→3e and produces a spectral shift of 17 nm. Luc-YG→3c, after removing members of luc-YG→YG' and members of the set which has a small effect on the spectra of luc-YG, is the set [S_{247},D_{352},S_{358}→G,V,T]. Hybrid 3c is shifted 20 nm from luc-YG. The sum of the effects of [R_{223},L_{238}→E,V] and [S_{247},D_{352},S_{358}→G,V,T] is 37 nm, about 10% greater than the spectral shift of luc-YG→OR. This is fairly good agreement, especially when acknowledging that luc-YG→OR has substitutions which contribute small effects that are not in either [R_{223},L_{238}→E,V] or [S_{247},D_{352},S_{358}→G,V,T]. Therefore, the effects of these two sets of mutations are largely additive.

Determining which amino acids are responsible for the green emission of luc-GR is a more difficult problem because the set of changes which describes this luciferase is so large. For example luc-YG→GR has 20 amino acid changes. The problem is compounded by the lack of one of the BstXI sites which have been so useful in analysing the other three luciferases. Hence, our analysis of luc-GR is so far only preliminary. The first two and last two variant positions have been found to have no affect on the spectrum of luc-GR, thus combining luc-GR→1a, luc-GR→1b, luc-GR→2a, and luc-GR→2b, gives luc-GR→GR' [V_9,L_{21},E_{403},I_{484}→I,K,K,K]. Luc-GR→4a, with luc-GR→GR' removed, gives [I_{323},R_{351},I_{389}→V,G,V], which shifts the spectrum of luc-GR 4 nm to 550 nm. Luc-GR→4b, with members of luc-GR→GR' removed, gives [D_{226},$V_{282}I_{283}$,R_{531}→E,IV,G] which shifts the spectrum to 553 nm. The difference between these two sets, i.e.

luc-4a→4b [D_{226},$V_{282}I_{283}$,V_{323},V_{389}→E,IV,I,I], shifts the spectrum 3 nm. This is a subset of a set identified earlier to have a 3 nm spectral shift in luc-YG→3d. Luc-GR→4c, after removal of luc-GR→GR', gives [S_{247},D_{266}, $V_{282}I_{283}$,R_{351},D_{352},S_{358}→G,E,IV,G,V,T] which shifts the spectrum 25 nm to 571 nm. This set can be divided into [D_{266},$V_{282}I_{283}$,R_{351}→E,IV,G] and [S_{247},D_{352},S_{358}→G,V,T]. The first set is the same as the effective set of luc-GR→4b, which gives a 7 nm shift, and the second set is the effective set of luc-YE→OR, which gives a 16 nm shift. Thus, the sum of these effects, 23 nm, is nearly the same as their combined affect. These observations are consistent with the interpretation that [I_{323},R_{351},I_{389}→V,G,V] can effect a 4 nm shift in the spectrum independent of other amino acid changes. As an additional example, luc-4e→3g, with removal of the amino acid changes shown to not affect the spectra, gives [L_{41},I_{323},R_{351},I_{389}→I,V,G,V], which causes a spectral shift 4 nm, from 577 nm to 573 nm. However, [I_{323},R_{351},I_{389}→V,G,V] can also be formed from luc-4d→YG with members of luc-YG→YG' removed. But in this case there is almost no change in the spectrum.

SUMMARY AND CONCLUSIONS

We have generated four different types of cDNA clones from the ventral light organ of *Pyrophorus plagiophthalamus*. These clones can direct the synthesis of luciferase in *E. coli* which are distinguishable by the colour of bioluminescence they emit. Since the different colours are expressible in a bacterial host, they cannot be due to post-translational modifications that are unique to beetles. Further, because of the many differences between prokaryotes and eukaryotes, it is unlikely that there are any post-translational modifications responsible for the different colours. Thus the determinants of colour must be found among the amino acid differences in the sequences of the four luciferases. We have begun to identify these determinants by constructing and analysing hybrid luciferases, made by recombining fragments of the four different types. The results have shown that there are two groups of amino acids that each can produce a greater than 16 nm change in the spectrum of a luciferase. There are at least two other groups of amino acids that can cause smaller changes in the spectra, and several amino acid which have virtually no effect on the spectra. The two sets of amino acid can be expressed as directional substitutions, [R_{223},L_{238}→E,V] and [S_{247},D_{352},S_{358}→G,V,T,], which in this form result in a large spectral shift towards longer wavelengths. It was shown that the effect of these two set of changes can act largely independent of each other. For the other sets of changes that cause smaller effects, the independence of their action is less clear.

It is not known by this analysis whether all amino acids in each set are required to effectuate a change. To determine this we are initiating site-directed mutagenesis to produce changes at only single sites. We anticipate that the amino acids producing the largest effect on the spectra will be those which have the largest changes in their chemical properties. Thus, in the set [R_{223},L_{238}→E,V], the effect is probably attributable mostly to R_{223}→E, which is a reversal of charge. In the set, [S_{247},D_{352},S_{358}→G,V,T], the effect is probably due to D_{352}→V, which changes a negative charge to a hydrophobic residue. But S_{247}→G may also be effective since it is a loss of hydrogen bonding.

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Complementary DNA Coding Click Beetle Luciferases Can Elicit Bioluminescence of Different Colors

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Eleven complementary DNA (cDNA) clones were generated from messenger RNA isolated from abdominal light organs of the bioluminescent click beetle, *Pyrophorus plagiophthalamus*. When expressed in *Escherichia coli*, these clones can elicit bioluminescence that is readily visible. The clones code for luciferases of four types, distinguished by the colors of bioluminescence they catalyze: green (546 nanometers), yellow-green (560 nanometers), yellow (578 nanometers), and orange (593 nanometers). The amino acid sequences of the different luciferases are 95 to 99 percent identical with each other, but are only 48 percent identical with the sequence of firefly luciferase (*Photinus pyralis*). Because of the different colors, these clones may be useful in experiments in which multiple reporter genes are needed.

NEARLY ALL OUR KNOWLEDGE OF beetle luciferases is derived from studies of a single species, the North American firefly *Photinus pyralis*. Comparative studies with other beetle luciferases have been hampered because of limited availability of the other species. Evolutionarily, beetle luciferases are unrelated to any of the other groups of luciferases that have been studied biochemically (*1*). Little is known about the luciferases from other beetles except that they all catalyze the production of various colors of light through the oxidative decarboxylation of beetle luciferin (*2*). Since the substrates of the luminescent reaction are the same in all these beetles, the different colors must be due to differences in the structure of the enzymes (*3*).

Recently we cloned a cDNA that codes for the luciferase of *P. pyralis*, and have shown that it can be used to express bioluminescence in *Escherichia coli*. We report here the cloning of cDNAs that code for several new luciferases from a bioluminescent click beetle, *Pyrophorus plagiophthalamus*. This beetle is unusual because it can emit bioluminescence of a wide range of colors from a single species. The expression products in *E. coli* of the cDNAs derived from this beetle are able to produce green, yellow-green, yellow, and orange light. As determined from the nucleotide sequences of the clones, the amino acid sequences of these click beetle luciferases are highly conserved among one another, but diverge from the sequence of the firefly luciferase. Taxonomy indicates that the click beetle luciferases probably are the most evolutionarily distant of the beetle luciferases from the firefly luciferase (*4*). This distance is reflected by differences in their chemical properties.

Pyrophorus plagiophthalamus is a large beetle with two sets of light organs. One set, on the dorsal surface of the head, emits light that is greenish but the exact color varies between individual beetles of the species, ranging from green (548 nm) to yellow-green (565 nm). The other set, at the anterior of the abdomen, generally emits light of a longer wavelength than the head organs but also varies between individuals ranging from green (547 nm) to orange (594 nm) (*5*). We converted mRNA isolated from the abdominal light organ of 60 beetles to cDNA and inserted this into a specialized lambda cloning vector, Lambda ZAP (*6*). The ability to convert this modified lambda vector into a bacterial expression plasmid (Bluescript) through an in vivo process allowed us to screen the cDNA library by two methods (*7*). In the phage form of the library, we screened with antibody to firefly luciferase that cross-reacts with the click beetle luciferases (*8*) and isolated four full-length clones that expressed bioluminescence in *E. coli*. A portion of the cDNA library was converted into the plasmid form, and we screened this for bioluminescence in the bacterial colonies. Bioluminescence can be initiated in colonies of *E. coli* expressing luciferase by adding luciferin to the media (*9*). Seven more cDNA clones were isolated by this method. It was determined visually that of the eleven clones, one produced green light, one produced yellow-green light, six produced yellow light, and three produced orange light.

Immunoblot analysis confirmed the production of full-length click beetle luciferase in *E. coli*. Despite some of these clones being detected with antibody to firefly luciferase during the library screening of plaques, we could not detect the gene products in blots made directly with *E. coli* lysates. The expression of bioluminescence was proved by transferring the cDNA cl into a plasmid vector incorporating th promoter (*10*). A lysate from *E. coli* expr ing the green-emitting luciferase from vector was partially purified. After gel-e trophoresis and blotting, a single antig band was revealed that comigrated with native click beetle luciferase. Subseque one cDNA clone from each of the f color-emitting groups was sequenced: open reading frame was revealed in each could potentially code a protein, the quence of which correlated with the en length of the sequence for firefly lucifer Thus the complete protein coding region the click beetle luciferases were apparer contained within their cDNA clones.

Expression of bioluminescence from *tac* vector yielded sufficient intensity, up addition of luciferin to the media, to all measurement of the spectral distributi from intact cells (Fig. 1). This confirmed visual assignment of the 11 cDNA clor into four color groups. For each of the fc colors, the bioluminescence spectrum is single peak qualitatively similar to the sp tra of the native click beetle luciferases (The range of colors from the clones representative of the full range measur from the abdominal light organs of livi beetles. However, there are colors emitt by the beetles, within the extremes of t range, that do not correspond to any of t clones (*5*). Thus other luciferase genes m not have been isolated. Spectra of the luci erases were also measured from partial purified preparations obtained from lysat of the *E. coli* expressing the cDNA clone

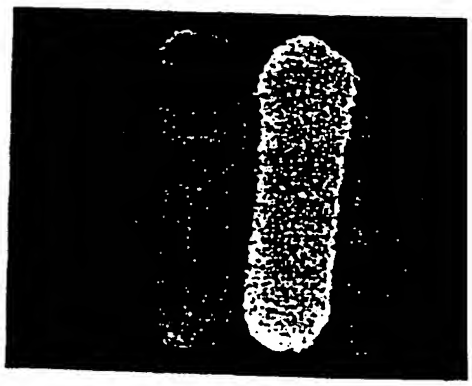

Fig. 1. Bioluminescence from colonies of *E. col* expressing the click beetle luciferases. Four streak of *E. coli*, each consisting of hundreds of colonies, show the four colors of bioluminescence emitted by the different luciferases. The colonies were grown on nitrocellulose filters layered on top of nutrient agar. To initiate the bioluminescent reaction, the filters were removed from the agar and soaked with 1 m*M* luciferin in 100 m*M* sodium citrate, *p*H 5.0. The photograph was produced from a 2-s contact exposure of the colonies onto Ektachrome 64.

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Between pH 6 to 7, the spectra of these preparations were indistinguishable from those of intact cells. At pH 8 there was a slight broadening of the spectra for the green- and yellow-emitting luciferases. The firefly luciferase shows a large spectral shift between pH 6 to 8. At pH 8 its spectral maximum is at 560 nm, which shifts to 615 nm (red) at pH 6 with a decrease in the quantum yield (*11*).

The sequences of the different click beetle luciferases are highly similar (Fig. 2). The open reading frame of each of the sequenced cDNA clones potentially codes a 543-residue polypeptide. Comparisons of the derived amino acid sequences show a 95 to 99% identity between the different color-emitting luciferases. Thus the number of amino acids that are responsible for the differences in the color is small. Because variation in color results directly from differences in the primary structures of the luciferases, specialized posttranslational modifications or unusual microenvironmental effects are not necessary to account for the color variation in the living beetles.

Comparison of the sequences of click beetle luciferases with that of firefly luciferase shows a low similarity. Alignment of their deduced amino acid sequences reveals that the various click beetle and the firefly luciferases are 48% identical (Fig. 3). Six gaps in the alignment of one to two amino acids in length account for most of a seven–amino acid difference in the lengths of the open reading frames between the firefly and click beetle luciferases. No regions in the alignment show especially high sequence similarity, thus giving little indication that particular regions have been conserved because of catalytic or structural constraints on the enzymes. An exception to this is in the last three amino acids which, for the firefly luciferase, have been shown to be necessary for translocation into peroxisomes (*12*). Given the close functional similarity of these enzymes, it is almost certain that the click beetle luciferases are also located in peroxisomes.

Firefly luciferase has historically been used as a bioluminescent reporter of chemical events associated with adenosine triphosphate (ATP) metabolism (*13*). With the cloning of its cDNA, this luciferase has also recently found application as an effective reporter of genetic events (*14, 15*). Its principal advantages are that (i) the initial polypeptide derived from the mRNA requires no posttranslational modifications for enzymatic activity; (ii) the luminescent reaction can be measured with high sensitivity; (iii) the assay of the gene product is rapid and does not use substrates requiring special precautions (such as radioactive isotopes or chemically unstable compounds); and (iv) gene expression may be detected without disruption of living tissue. Compared with the conventionally used assay of chloramphenicol acetyltransferase (CAT) for gene activity, firefly luciferase is assayed in minutes as opposed to hours, and is 100 to 1000 times more sensitive (*15*).

The cDNAs coding for the click beetle luciferases also have these features, and, as they can be distinguished by color, may be useful in situations where multiple reporters are desirable. Expression in exogenous hosts should differ little between these luciferases because of their sequence similarity. Also, since the colors do not shift near physiological pH, the different luciferases can be distinguished in vivo as well as in vitro. Thus the click beetle luciferases may provide a dual reporter system that can allow two different promoters to be monitored within a single host, or for different populations of

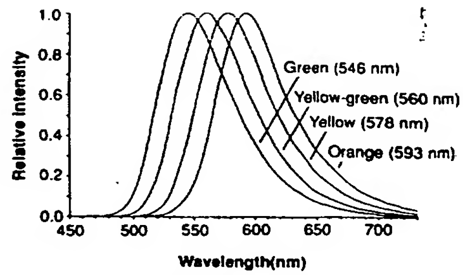

Fig. 2. Spectra of the click beetle luciferases, from intact *E. coli* immersed in luciferin, show four overlapping peaks of nearly even spacing. The maximum intensity of each spectrum has been normalized. The spectra were measured on a Fastie-Ebert–type grating spectrometer (*2*) and were corrected for the spectral sensitivity of the photomultiplier tube and for time-dependent variation in the intensity of the luminescent reactions. Wavelength positions were calibrated against the spectral lines of a mercury vapor lamp. Virtually identical spectra were obtained from lysates of these *E. coli* between pH 6 to 8. The lysates were prepared from cells grown to middle or late log-phase growth at 37°C, and then for 2 hours at 30°C with isopropylthiogalactoside (IPTG) added to 1 m*M*. The cells were washed and resuspended in approximately 1/150 volume of the culture with 100 m*M* potassium phosphate, pH 7.0, 2 m*M* EDTA, and 35% of saturation $(NH_4)_2SO_4$. After lysis by sonication and removal of the debris by centrifugation, $(NH_4)_2SO_4$ was added to 53% of saturation and the precipitate was dissolved in 1/15 volume of the lysate with 100 m*M* potassium phosphate, pH 7.0, 2 m*M* EDTA, and 50% glycerol. The spectra were measured from 10 µl of this solution diluted 100-fold with 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid, 50 m*M* 2-(*N*-morpholino)propanesulfonic acid (MOPS), 50 m*M* tricine, 5 m*M* $MgSO_4$, 1 m*M* EDTA, 0.1 m*M* luciferin, 1.5 m*M* ATP, 1 m*M* NaF, 0.2 mg of bovine serum albumin per milliliter, and 10% glycerol. The spectra were measured at pH 6.0, 7.0, and 8.0.

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FF   .EDA..IKK..A.FY....G....Q.HK.MKRYALV.GTI.FT.AHI.VN.T.A.Y..MSVR..EAMKRY.LNTNHRIVV.S..SLQ..M.   91
GR  MMKREKNVVYGPEPLHPLEDLTAGEMLFRALRKHSHLPQ--ALVDVYGEEWISYKEFFETTCLLAQSLHNCGYKMSDVVSICAENNKRFFVP   90
YG  ........I..............................  .....F.D.SL......A...............N..............I.   90
YE  ........I..........K...................  .I...F.D.SL......A...............N................   90
OR  ........I..........K...................  .I...F.D.SL......A...............N..............I.   90

FF  VLG.LF..VA...A.DI.NER..LNS.N..Q.TV..VS.KG.Q.I.N..KKLPI.QK...M.SKTDYQ.FQ.MYT.VT.HLPP.F.EYD.V.  183
GR  IIAAWYIGMIVAPVNEGYIPDELCKVMGISRPQLVFCTKNILNKVLEVQSRTDFIKRIIILDAVENIHGCESLPNFI-SRYSDG-NIANFKP  180
YG  ................S............K..I..................N.........T............ ...... .......  180
YE  ................S............K..I..................N.........T............ ...... .......  180
OR  ................S............K..I..................N.........T............ ...... .......  180

FF  ESF.RDKTI.L.MN...S.......ALP..TA...FS..R..IF.N.I..DTAI.SVV..H.G..MFTT...LIC.F..VLMY..EE.L..R  275
GR  LHYDPVEQVAAILCSSGTTGLPKGVMQTHRNVCVRLIHALDPRVGTQLIPGVTVLVYLPFFHAFGFSINLGYFMVGLRVIMLRRFDQEAFLK  272
YG  ............................Q.I..........A..................................................  272
YE  ............................Q.I.........EA............V..........................E......  272
OR  ............................Q.I.........EA............V.......G..................E......  272

FF  SL...KIQ.ALL..TLFS.FA..T.I......N.H.IAS.G...S...G.AVA..FH.....Q.Y....T...ILITPEGDD.P.AV.K.V.  367
GR  AIQDYEVRSVINVPAIILFLSKSPLVDKYDLSSLRELCCGAAPLAKEVAEIAVKRLNLPGIRCGFGLTESTSANIHSLRDEFKSGSLGRVTP  364
YG  ..............................................V..............................G...........  364
YE  .........IV...................................................................G...........  364
OR  .........IV...................................................................G...........  364

FF  FFE..VV.LD...T..V..R....VR...IMS.....P...NAL..K........IA.W......FI...L.S......Y........S...  459
GR  LMAAKIADRETGKALGPNQVGELCIKGPMVSKGYVNNVEATKEAIDDDGWLHSGDFGYYDEDEHFYVVDRYKELIKYKGSQVAPAELEEILL  456
YG  ......................V.....................................................................  456
YE  ............................................................................................  456
OR  ..............................K.............................................................  456

FF  QH.N.F.AG.A.L..DD.....A.V..LEH..TM.E..IV..V.SQ.TTA.K.....V...EV.KGL...LDA..IR.I.I.AK.GGK...  550
GR  KNPCIRDVAVVGIPDLEAGELPSAFVVIQPGKEITAKEVVDYLAERVSHTKYLRGGVRFVDSIPRNVTGKI-TRK--E-LLKQLLEKSSKL  543
YG  ........................K.................................................................  543
YE  ......................................................................... ...  . ...........  543
OR  ......................................................................... ...  . ...........  543
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Fig. 3. Alignment of the amino acid sequences of the click beetle and the firefly luciferases is shown to emphasize sequence differences. The sequence information is derived from the open reading frames of the corresponding cDNA clones. The identity of each luciferase sequence is indicated at the right of each line by a two letter code: FF, firefly; GR, green-emitting click beetle; YG, yellow-green–emitting click beetle; YE, yellow-emitting click beetle; and OR, orange-emitting click beetle. Only the sequence for the green-emitting click beetle luciferase is shown in entirety. Gaps in the alignment of this sequence are indicated by hyphens. Other luciferase sequences have letter designations only at sites where they differ from the green-emitting luciferase; where the sequences are the same there is a period. Numbers on the right indicate the position of the amino acid at the end of each line. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

cells to be observed simultaneously. The ability to distinguish each of the luciferases in a mixture, however, is limited by the width of their emissions spectra. From calculations based solely on the overlap of the spectra of the green- and orange-emitting luciferases, one luciferase in a mixture should be detectable in the presence of a 25-fold excess of the other.

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6 December 1988; accepted 7 March 1989

Reexamination of the Three-Dimensional Structure the Small Subunit of RuBisCo from Higher Plants

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The structure of L_8S_8 RuBisCo (where L is the large subunit and S is the sn subunit) from spinach has been determined to a resolution of 2.8 ångstrom by usi fourfold averaging of an isomorphous electron density map based on three heavy-at derivatives. The structure of the S subunit is different from that previously reported the tobacco S subunit in spite of 75 percent sequence identity. The elements secondary structure, four antiparallel β strands and two α helices, are the same, but t topology and direction of the polypeptide chain through these elements dif completely. One of these models is clearly wrong. The spinach model has hydrophol residues in the core between the α helices and β sheet as well as conserved residues the subunit interactions. The deletion of residues 49 to 62 that is present in t *Anabaena* sequence removes a loop region in the spinach model. The positions of thi mercury atoms in the heavy-atom derivatives agree with the assignment of side chai in the spinach structure.

CHAPMAN *et al.* (*1*) HAVE RECENTLY described the tertiary structure of plant RuBisCo, the key enzyme (*2*) in the Calvin cycle of carbon dioxide fixation in photosynthesis. Their model is based on an electron density map to 2.6 Å of the L_8S_8 molecule from tobacco. We have determined the structure of L_8S_8 RuBisCo from spinach to 2.8 Å resolution and find very significant differences in the structure of the S subunit compared with the reported tobacco structure. Since there is 75% identity between the amino acid sequences of these two polypeptide chains, they are expected to have similar tertiary structures.

Crystals of spinach RuBisCo that diffract to 1.7 Å resolution were grown from solutions of the activated form of the enzyme with a bound transition-state analogue (*3*). These crystals contain one-half the L_8S_8 molecule in the asymmetric unit. There is a local noncrystallographic fourfold axis through the molecule, which has approximate 422 symmetry. X-ray data were collected on the synchrotron radiation source in Daresbury, United Kingdom, for the native enzyme and three heavy-atom derivatives. An initial electron density map was calculated with the use of isomorphous phase angles. These were refined by real-space averaging (*4*) around the local fourfold axis. Data collection procedures and phasing statistics have been briefly described (*5*).

The final electron density map was of very good quality, as would be expected by fourfold averaging of an electron density map based on three heavy-atom derivatives. A most all of the side chains could easily identified from the known sequences of t spinach S and L chains (*6*, *7*), which co prise 123 and 475 residues, respective The sequence of the S subunit, which w determined by amino acid analysis (*6*), co tains only one Cys residue, Cys 112. Ho ever, two independent determinations of t amino acid content of the spinach sm subunit (*8*) made in different laboratori have shown that there are three Cys residu per subunit. Furthermore, almost all of t small subunits from higher plant RuBisC for which the sequences are known conta three Cys residues at positions 41, 77, ar 112. We therefore conclude that in all prol ability the spinach small subunit also co tains Cys residues at these three position Our electron density map also strongly su ports Cys side chains at these positions; th side-chain electron densities are appropria for Cys (Fig. 1b).

We first built the L chain (*5*) using th known structure of L_2 RuBisCo from *Rh dospirillum rubrum* (*9*). We found, in agre ment with the work on the tobacco enzym (*1*, *10*), that higher plant L chains have structure that is quite similar to that of th bacterial enzyme (*9*) except at the carboxy terminal. The arrangement of the L subunit in the spinach enzyme into four L_2 dimer

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Introduction to Beetle Luciferases and their Applications

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All beetle luciferases have evolved from a common ancestor: they all use ATP, O_2, and a common luciferin as substrates. The most studied of these luciferases is that derived from the firefly *Photinus pyralis*, a beetle in the superfamily of Cantharoidea. The sensitivity with which the activity of this enzyme can be assayed has made it useful in the measurement of minute concentrations of ATP. With the cloning of the cDNA coding this luciferase, it has also found wide application in molecular biology as a reporter gene. We have recently cloned other cDNAs that code for luciferases from the bioluminescent click beetle, *Pyrophorus plagiophthalamus*, in the superfamily Elateroidea. These newly acquired luciferases are of at least four different types, distinguishable by their ability to emit different colours of bioluminescence ranging from green to orange. Unique properties of these luciferases, especially their emission of multiple colours, may make them additionally useful in applications.

Keywords: Firefly luciferase; click beetle luciferases; reporter genes

INTRODUCTION

Man's perception of the world is visually oriented. Since bioluminescence is one of the few things that can be seen in the dark, it is understandable that this has been a topic of biochemical research for many decades. Fireflies have been prominent in this research endeavour, in part because they are abundant and their light organs are replete with luciferase. Thus they provided a plentiful resource for further study. Early research on fireflies was done primarily to better understand this peculiar phenomena of living light. Sometimes, though, the earliest work was justified as a means of developing artificial lighting. In the late 1940s, when the general importance of ATP metabolism was just becoming recognized, it was discovered that ATP was a component in the luminescent reaction of fireflies. The firefly luciferase became one of the paradigms of ATP-utilizing enzymes. Because of the extreme sensitivity with which the activity of this enzyme could be assayed, it was soon adapted as a tool in the measurement of very low concentrations of ATP. Subsequently, luciferase was combined with other ATP-utilizing enzymes to produce coupled enzymatic systems. In these systems, the luciferase was the reporter allowing sensitive measurements of a wide variety of metabolites.

Recently, firefly luciferase has found new application as a reporter of genetic activity in

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living cells. Its application in this area was made possible by the cloning of its cDNA, which can direct the synthesis of active enzyme in foreign hosts (de Wet *et al.*, 1985). In this report, we describe briefly how the properties of luciferase have made it well suited for this purpose. We also present information on the recent cloning of several new cDNAs from a bioluminescent click beetle (Wood *et al.*, 1988a). These cDNA clones encode for four different types of luciferases, which can be distinguished by their ability to emit different colours of bioluminescence. These colours range from green to orange. Structurally, the click beetle luciferases differ significantly from the firefly luciferase, and these differences are reflected in their chemical properties. Because of this, the click beetle luciferases may have additional features to make them useful as genetic reporter.

PROPERTIES OF THE FIREFLY LUCIFERASE

All beetle luciferases catalyse the conversion of chemical energy into light by a two-step process (Fig. 1)(Seliger and McElroy, 1964; DeLuca and McElroy, 1978). This process utilizes ATP, O_2, and beetle luciferin, a unique heterocyclic acid found only in bioluminescent beetles. In the first step, the carboxylate group of luciferin is activated by acylation with the alpha-phosphate of ATP. The luciferyl adenylate is then oxidized with molecular oxygen, in the second step, to yield AMP, carbon dioxide, and oxyluciferin. The oxyluciferin is generated in an electronically excited state which, upon transition to the ground state, emits the photon characteristic of bioluminescence. For firefly luciferase, the most studied of the beetle luciferases, the quantum yield for this reaction has been measured at 0.88 relative to the consumption of luciferin (McElroy and Seliger, 1960). This is the highest yield reported for any luminescent reaction.

Under optimal conditions the firefly luciferase emits light whose peak intensity is at 561 nm (yellow-green). This is the same as the light emitted from live fireflies. Under a variety of conditions, however, the structure of luciferase can be altered to a form which emits predominately at 617 nm (red) (Seliger and McElroy, 1964). Some conditions which can cause this spectral shift are pH below 7.5, temperature above 20 °C, the presence of denaturants such as urea, and the presence of heavy metals such as Zn^{2+}, Cd^{2+}, or Hg^{2+} (Seliger and McElroy, 1964). Some chemical modifications of the enzyme, or the use of substrate analogues, can also cause the enzyme to emit red light (DeLuca *et al.*, 1973; Alter and Deluca, 1986). In the case of pH, the shift to red light is associated with a substantial decrease in the quantum yield of the reaction (McElroy and Seliger, 1966). This decrease in quantum yield is probably evident under any condition that promotes the red-emitting form. The spectral shift associated with changes in temperature, or the presence of denaturants, can be interpreted as resulting from partial unfolding of the enzyme structure. For others conditions, it is not known whether the effects are localized to key reactive residues, or whether they also cause general perturbations to the structure. Aside from the actual decrease in the quantum efficiency of luciferase in the red-emitting form, the spectral shift also causes an apparent decrease in enzymatic activity. This is because photomultiplier tubes are generally much less sensitive to red light than green light. Both these real and apparent affects combine to give a large pH dependence to the measured light output of firefly luciferase. The optimal pH for light output is pH 7.8.

Under conditions of excess substrates, the light output of luciferase is proportional to the

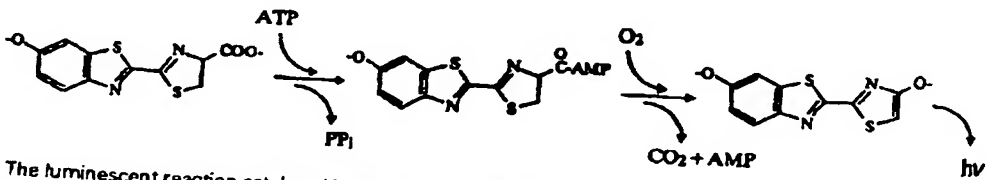

Figure 1. The luminescent reaction catalysed by beetle luciferase. Beetle luciferin is the heterocyclic molecule depicted on the left. Other reactants are depicted by their common abbreviations. The final product, *hν*, is the photon of light emitted from oxyluciferin during its transition to its ground-state electronic configuration

concentration of enzyme over at least a 10,000-fold range. With the use of a sensitive luminometer, as little as 10 femtograms of enzyme can be detected (10^5 molecules). Initiation of the luminescent reaction by rapid mixing of the substrates with the enzyme results in a rapid release of light which reaches maximum intensity after about 0.3 seconds. The intensity is then quickly inhibited in a biphasic manner, reaching about 10% of its peak value after 30 seconds. Beyond one minute the intensity is produced from a steady-state process which decays slowly over several hours. Because the peak luminescence is typically over 10-fold greater than the steady-state luminescence, the enzymatic assay is most sensitive when the first 30 seconds of the reaction are included in the measurement of total light output. This is typically accomplished in a luminometer where the reaction is initiated in front of the photomultiplier tube by injection of substrates directly into the sample holder.

Firefly luciferase is a 61 kD enzyme which apparently is active as a monomer. It is coded by a 550 amino acid reading frame in its mRNA, and is probably produced as an active enzyme without the necessity of post-translational modifications. In the firefly, the enzyme is located in specialized peroxisomes of the light organ (Keller *et al.*, 1987). It is directed to these sub-cellular organelles by a targeting sequence at the C-terminus of the protein (Gould and Subramani, 1988b, 1988c). This targeting sequence is conserved throughout eukaryotes, and will cause the luciferase to localize in the peroxisomes of other organisms when expressed in exogenous hosts.

APPLICATION OF FIREFLY LUCIFERASE AS A REPORTER GENE

With the past decade have come dramatic advancements in our ability to manipulate genetic materials. Enabled by these new techniques, the study of sub-cellular events which regulate genetic activity has become one of the largest areas of research today. A key tool in this area of research is the reporter gene, which provides an observable parameter in the monitoring of genetic events at a molecular level. In its simplest form, a reporter gene is a fragment of DNA which encodes for an easily detectable protein. This protein is the reporter. In experiments, the reporter gene is linked to other fragments of DNA which are thought to contain genetic control elements, and the assemblage is introduced into living cells. Production of the reporter in the cell is regulated by the action of the control elements on the transcriptional activity of the reporter gene. Thus, the reporter is the observable parameter allowing the experimenter to monitor the action of the control elements.

In practice, the transcriptional activity of a reporter gene can be quite low, and experiments are often limited by an inability to detect the reporter. Therefore, for a reporter to be widely useful, it must be detectable in very low concentrations. In addition, the reporter must be detectable by a method that can distinguish it from other proteins native to the host cell. Firefly luciferase meets these criteria ideally. It can be detected in very small amounts through its biolumninescent activity, and since bioluminescence is not a common event in living systems, its activity will be unique in the experimental host. That is, there is no endogenous luminescent activity of the host to interfere with the detection of even miniscule amounts of luciferase. The bacterial enzyme, chloramphenicol acetyltransferase (CAT), has been used conventionally as a reporter in eukaryotic systems for similar reasons. Its enzymatic activity is not found in eukaryotic cells, so CAT can also be detected without confusion from host actitivities. Its assay is based on conversion of the antibiotic chloramphenicol to mono- and di-acetylated forms. High sensitivity is provided by the use of ^{14}C-labelled chloramphenicol as the substrate. This method requires that the products of the reaction be separated from the substrate before quantification, usually by thin layer chromatography or HPLC.

Because CAT is widely used as a genetic reporter, it was used as a benchmark to evaluate the suitability of firefly luciferase in this application (de Wet *et al.*, 1987). It was found that the levels of expression of CAT and luciferase in eukaryotic systems were comparable. It had been previously shown that the production of CAT in eukaryotic cells is proportional to mRNA transcription from the reporter gene. Since luciferase production paralleled CAT production under a variety of experimental conditions, luciferase must also be a proportional indicator of transcriptional activity. However, because of the efficient detection methods achievable with bioluminescence, the assay of luciferase is 100 to 1000 times

more sensitive. Thus, much lower levels of genetic activity are detectable. Furthermore, the time required to assay luciferase is much less than CAT. Using a luminometer or scintillation counter, the luciferase assay requires about a minute per sample. The CAT assay usually requires several hours. The assay of luciferase also does not require the special precautions needed for radioactive ^{14}C.

One of the unique advantages of firefly luciferase as a reporter of genetic activity is its potential to measure this activity from within living cells. This is not possible with use of CAT since the products of the reaction require separation from the assay mixture in order to be quantified. The photons produced in the luciferase reaction, however, are generally able to pass from within the host cell to allow external detection. A precondition of this is that the luciferin substrate be able to pass into the cell to combine with the luciferase reporter. The other substrates of the reaction, ATP and O_2, are readily available in the interior of the cell. The mere addition of luciferin to the external media is sufficient to allow its passage across the cellular membrane. But the light output elicited by this method is less than expected given the extent of luciferase contained within the cells. Light output can be increased with the use of permiablizing agents such as DMSO or nigericin (Gould and Subramani, 1988a), but still not to the full potential expected. It is not known whether permeability of the outer membrane is the only limitation, or whether there are other inhibitors of activity. One possibility is that the peroxisomal membrane acts as a second barrier to luciferin passage. Since luciferase is localized into peroxisomes, most of the luminescent activity may arise from these organelles. Experiments are currently under way to remove the peroxisomal targeting signal from luciferase so that it will remain in the cytoplasm. This may improve its ability to elicit luminescence from within intact cells. However, other possibilities exist, such as unfavourable microenvironmental effects, which could inhibit the activity of luciferase in a foreign host.

Since the first published reports of its use as a genetic reporter, this new application of firefly luciferase has received much interest. By the time this article was written, we had received approximately 1000 requests for the cDNA encoding luciferase from other laboratories wishing to apply it to their experimental systems. The feedback from these other laboratories has been quite positive. In most cases, researchers are finding that the use of luciferase instead of CAT is saving much time in the execution of their experiments. The time saved is not only in the much shorter assay time of luciferase, but also in the time required for sufficient expression of the reporter. Previously, production of the reporter often was not detectable for 24 to 48 hours after the reporter gene was introduced into cells. Because of the much higher sensitivity of the luciferase assay, expression of the reporter gene can typically be measured after only a few hours. In some cases, where expression of the reporter was previously too low for detection under any conditions, the use of luciferase has allowed measurements to be made. To date, luciferase has been expressed from its cDNA in almost every living kingdom. It has been expressed in bacteria (de Wet *et al.*, 1985), yeast (Wood and DeLuca, 1987), dictyostelium (Howard *et al.*, 1988), mammalian cells (de Wet et al., 1987, Gould and Subramani, 1988a), and plant cells (Ow *et al.*, 1986), as well as in transgenic mice (DiLella *et al.*, 1988; Crenshaw and Rosenfeld, 1988) and plants (Ow *et al.*, 1986).

COMPARISON OF FIREFLY AND CLICK BEETLE LUCIFERASES

Bioluminescent beetles are found in two superfamilies, Elateroidea and Cantharoidea (Lloyd, 1978). Fireflies are members of the family Lampyridae in the superfamily Cantharoidea; as indicated above, they have been the primary source of a beetle luciferase because of their abundance and accessibility. In the superfamily Elateroidea, only the family Elateridae contains bioluminescent members, which are more commonly known as click beetles. This family of beetles is one of the most widely distributed, with species found in most areas of the world. However, unlike Lampyridae, where nearly all of the members are bioluminescent, only a small percentage of Elateridae are so. Most of these are located in the Caribbean and in South America. Their taxomony suggests that the click beetles are the most evolutionarily distant of the bioluminescent beetles from the fireflies (Crowson, 1981). The time of divergence of the Elateroidea and Cantharoidea superfamilies cannot be estimated directly owing to a lack of fossils. But by

comparison of the morphological differences between these groups of beetles, corroborated with the fossil record of other beetles, it has been estimated that these superfamilies diverged about 120 million years ago.

Morphologically the click beetles and fireflies are quite distinct (Fig. 2). The click beetles have a hard exoskeleton, and are often larger than the fireflies. They can be recognized by a characteristic behaviour they display when being constrained or placed on their backs. They make an audible 'click' sound by forcibly arching their head forward. Bioluminescent click beetles have two sets of light organs. One pair is located on the dorsal surface of the head. These light organs emit long pulses of light when the beetles are not in flight. The second set is a single organ located in a cleft on the ventral surface of the beetle between the mesothorax and abdomen. This light organ also emits long pulses of light but only when the beetle is in flight. On the ground the cleft is closed and the light is extinguished. For most species of bioluminescent click beetle, the ventral organ emits light at a longer wavelength than the dorsal organ. The position and activity of the light organs in fireflies is quite different. These beetles have one set of light organs located on the ventral surface of the abdomen, on the posterior sternites. They generally emit short burst of light in a pattern which is indicative of the particular species.

One species of click beetle has been of particular interest since its bioluminescence was first studied in 1963. This species, *Pyrophorus plagiophthalamus*, is an especially large click-beetle being typically 3 cm in length. It was of interest because its bioluminescence presents an unusually large range of colorus (Seliger *et al.*, 1964). Furthermore, the colours vary between individuals, a property not found in fireflies. The light of the dorsal organ is greenish in colour, but varies between individuals from green (548 nm) to yellow-green (565 nm). The ventral organ varies over a much wider range, from green (547 nm) to orange (594 nm) (Biggley *et al.*, 1967). The luciferases of these beetles were extracted to determine the source of these different colours. In extracts, the bioluminescence spectra were not different from those of the living beetles. Analysis showed that the different colours were not due to alterations of the substrates of the reaction, which are the same as utilized by the firefly luciferase. It was concluded that the differences were due to variation in the interaction of the substrates with the luciferases (Seliger and McElroy, 1964). Unfortunately, attempts to study these luciferases further were limited by the difficulty of collecting sufficient quantities of the beetles.

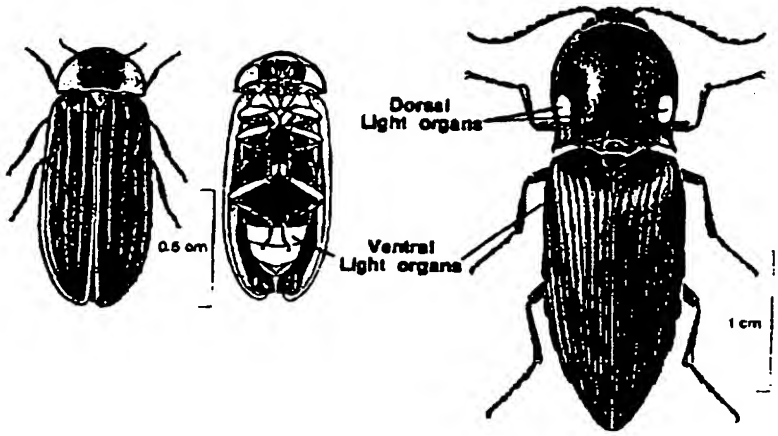

Figure 2. The general morphology of fireflies and click beetles. A firefly (Lampyridae) is shown in two views on the left, and a click beetle (Elateridae) is shown on the right

CLONING AND EXPRESSION OF CDNAS ENCODING CLICK BEETLE LUCIFERASES

Expression of firefly luciferase in *Escherichia coli* demonstrated that we could produce this enzyme from a source which was easily grown in the laboratory. Thus, in this case, the information contained in the cDNA encoding firefly luciferase was in itself sufficient to generate an active enzyme in a foreign host. Application of this technology to the click beetle luciferases could circumvent the problems of collecting large quantities of the beetles. The methods used in cloning a cDNA which encodes luciferase require only a small supply of the beetles, and they are needed only once. Afterwards, bacterial hosts generate the DNA and enzyme needed for further study. Production of the click beetle luciferases from cDNA clones has the additional advantage that genetic variants of the enzyme, such as those which produce the different colours of bioluminescence, are generated in isolation of one another. Enzymes isolated from the click beetles directly would require methods for separation of the different variants. This would be difficult since, as was subsequently found, the physical differences between these variants are few. Furthermore, the amino acid sequences of the luciferases can be determined from the DNA sequences of their cDNA clones. DNA sequencing is an efficient technique making it practicable to determine the amino acid sequence differences between several proteins of over 500 amino acids each.

Specimens of *Pyrophorus plagiophthalamus*, collected from the northeast end of Jamaica, were transported live to the laboratory and frozen in liquid N_2. Messenger RNA was isolated from ventral light organs of approximately 60 beetles, one microgram of which was converted to cDNA (de Wet *et al.*, 1986). This was packaged in a specialized lambda cloning vector, Lambda ZAP, to yield 700,000 recombinant plaques (Fig. 3) (Short *et al.*, 1988). We had originally intended to screen the library by DNA hybridization using the cDNA sequence of firefly luciferase. However, attempts at visualizing the click beetle luciferase gene in Southern blots, using the firefly luciferase cDNA as the probe, failed to demonstrate cross-hybridization even under conditions of low stringency. It had been previously shown that antibodies raised against firefly luciferase can cross-react with the click beetle luciferases

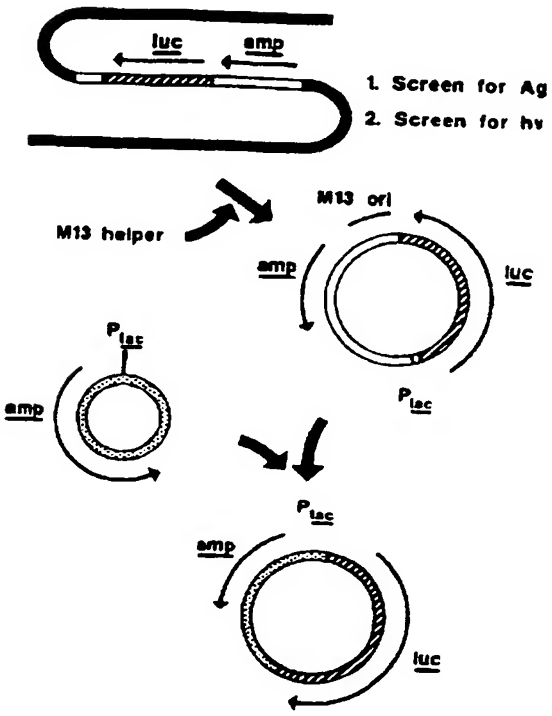

Figure 3. The strategy for cloning and expressing the cDNAs coding click beetle luciferases. The upper left corner depicts the Lambda ZAP vector. The cDNA library in this form was screened for the expression of antigenic polypeptides (Ag). With the use of M13 helper, Lambda ZAP was transformed either individually or *en bloc* into Bluescript plasmids (upper right). The cDNA library in this form was screened for the expression of luminescence (*hv*). The expression of luminescence was improved by transferring the cDNA clones to a vector containing the *tac* promoter (lower left and bottom)

(Wienhausen and DeLuca, 1985). Thus we used such antibodies, raised in rabbits, to screen the cDNA library. Because the cross-reactivity with click beetle luciferases is weak, we used antibodies that had been affinity purified by selection on a Sepharose column containing immobilized firefly luciferase (a gift from Dr Gilbert Keller; Keller *et al.*, 1987).

The original screening was done on unamplified aliquots of the lambda cDNA library. Determined by the colorimetric detection of alkaline phosphatase conjugated to anti-rabbit antibodies, 5.5% of the recombinant lambda phage expressed luciferase antigens. Eighteen clones were chosen for further analysis. A unique

feature of the Lambda ZAP cloning vector is that it can be transformed into a bacterial expression plasmid (Bluescript) by an *in vivo* process involving the addition of an M13 helper phage (Short *et al.*, 1988). The recombinant lambda containing the four longest cDNA clones were transformed into their plasmid form, and found to be able to express bioluminescence in *E. coli*. It could be visually observed from the expression of the four clones in *E. coli* that two produced orange light, one produced yellow light, and one produced yellow-green light.

In order to ascertain whether other colours of bioluminescence could also be found in the library, it was rescreened for other full-length cDNA clones. The rescreening was done by a different method designed to identify luminescent activity directly. Five aliquots of the original library were amplified, then transformed *en bloc* into expression plasmids. As in the case of eukaryotic cell expressing the firefly luciferase (see above), bioluminescence can be initiated in *E. coli* expressing luciferase by the addition of luciferin to the media (Wood and DeLuca, 1987). In bacteria, the diffusion of luciferin through the membranes can be facilitated by reducing the pH of the media to 5. Presumably this masks negative charges on the molecule, making it more hydrophobic and permeable to a lipid bilayer. By adding luciferin to bacterial colonies containing clones of the cDNA library, colonies able to express a functional luciferase were identified directly by their ability to darken X-ray film. Several bioluminescent colonies were isolated from each aliquot of the library, seven of which were identified as arising from independent cDNA clones. From two of the aliquots, two colonies could be judged as resulting from independent clones based on widely different intensities. The independence of these clones was later confirmed by restriction mapping. From these clones, five emit yellow light, one emits orange light, and one emits a new colour, green.

Western blot analysis was performed to confirm that full-length click beetle luciferases were being expressed in the *E. coli*. Despite the fact that some of these clones were clearly visualized by anti-firefly luciferase antibody during the library screening, we were unable to detect the gene products in blots made directly with *E. coli* lysates. This is the result of both a low level of gene expression, and a weak cross-reactivity with the antibody. The expression of luminescence was increased by transferring the cDNA clones to a plasmid vector which incorporated a *tac* promotor (Fig. 3). A lysate from *E. coli* expressing the green-emitting luciferase from the *tac* vector further required partial purification to be detectable in a blot. The blot revealed a single band, cross-reactive with firefly luciferase, which comigrates with the native click beetle luciferase (Fig. 4). DNA sequence analysis was later performed

Figure 4. Western blot showing the expression of click beetle luciferase in *E. coli*. Lane 1: partially purified extract of *E. coli* expressing the green-emitting luciferase. Lane 2: extract of click beetle light organ. Lane 3: purified firefly luciferase. Luciferases were detected with anti-firefly luciferase

for one clone of each colour. This confirmed that each cDNA contained an open reading frame which could code for a protein whose N-terminus corresponded to the N-terminus of firefly luciferase. Thus, as has been achieved previously with the firefly luciferase, the click beetle luciferases can be produced in *E. coli* as full-length and enzymatically active enzymes.

BIOLUMINESCENCE SPECTRA OF CLICK BEETLE LUCIFERASES

Spectrographic analysis was performed on the bioluminescence emitted from *E. coli* expressing the various cDNA clones. Bioluminescence was induced from whole cells by the same method used previously in the screening of bacterial colonies for luminescence. Cells producing luciferase from the *tac* vector yielded sufficient light intensity, upon addition of luciferin to the media, to allow spectral measurements. These measurements verified the visual observation that the eleven clones can be sorted into four groups based on the colour of light emitted. For each of the colours, the spectrum is a single peak qualitatively similar to the spectra of native click beetle luciferase (Seliger *et al.*, 1964). When the spectra of the four colours are superimposed, they show a remarkable pattern of four similarly shaped peaks that are nearly evenly spaced (Fig. 5). The wavelengths of maximum intensity are 546 nm for green, 560 nm for yellow-green, 578 nm for yellow, and 594 nm for orange.

Spectra were also measured from lysates of the *E. coli* after partial purification (Wood *et al.*, 1988a) (Fig. 6). Bioluminescence was elicited from the lysates by diluting them 100-fold into a reaction mixture ranging in pH from 6 to 10. For pHs 6.0, 7.0, and 8.0, the reaction mixture was buffered with 50 mmol/l MES/50 mmol/l MOPS/50 mmol/l Tricine. For pHs 8.0, 9.0, and 10.0 it was buffered with 50 mmol/l Tricine/50 mmol/l CHES. Also in the reaction mixture were 5 mmol/l $MgSO_4$/1 mmol/l EDTA/0.1 mmol/l luciferin/1.5 mmol/l ATP/1 mmol/l NaF/0.2 mg/ml BSA/10% glycerol. (NaF was found to simplify the kinetics of the decay of luminescence, which simplified the analysis of the spectral data. It does not affect the spectral distribution. It is not known whether it affects the activity of the click beetle luciferase directly, or whether it is due to an interaction with other components of the lysate. It has no effect on the purified firefly luciferase.) For the click beetle luciferases from each of the four colours, the spectra measured from whole cells matched that of the lysates at pH 6.0 and pH 7.0. Also, for each of the luciferases, the spectra shifted towards longer wavelengths at pH above 9.0. This shift was largest for the green-emitting luciferase, less for the yellow-emitting luciferase, and the least for the yellow-green and orange-emitting luciferase. At pH 8.0, this shift is virtually undetectable for the yellow-green and orange-emitting luciferases. For the green and yellow-emitting luciferases, the shift at pH 8.0 can be detected as a slight widening of the spectral peak, but the position of the maxima is unchanged.

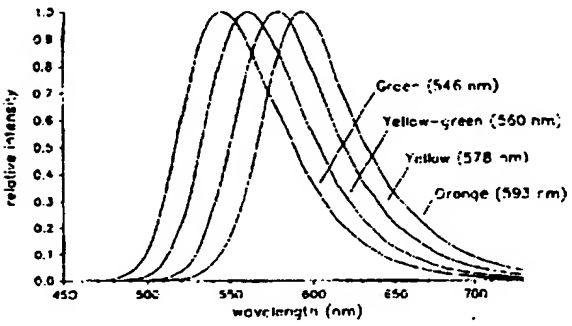

Figure 5. Spectra of bioluminescence emitted from *E. coli* cells containing the click beetle luciferases. The intensity maximum for each spectrum has been normalized

This pH response of the click beetle luciferases is in contrast with that of the firefly luciferase. As stated above, the spectrum of firefly luciferase shifts to longer wavelengths at low pH (Fig. 7). In the pH range of 8.0 to 10.0, the enzyme emits its characteristic yellow-green light. The spectrum shifts towards longer wavelengths at pH 7.0, and at pH 6.0 is generated almost completely from a red-light emitting form of the enzyme. This shift is much larger than is seen with the click beetle luciferases. At pH 7.0, where the spectrum of firefly luciferase is a mixture of yellow-green and red-emitting forms of the enzyme, a difference is apparent between the enzyme purified from fireflies and that produced in *E. coli* (Fig. 7). With the luciferase from *E. coli*, the red component of the spectrum is much less than for the purified native enzyme. In addition, as the light output of the reaction decays, the two components of the spectrum do not decay at the

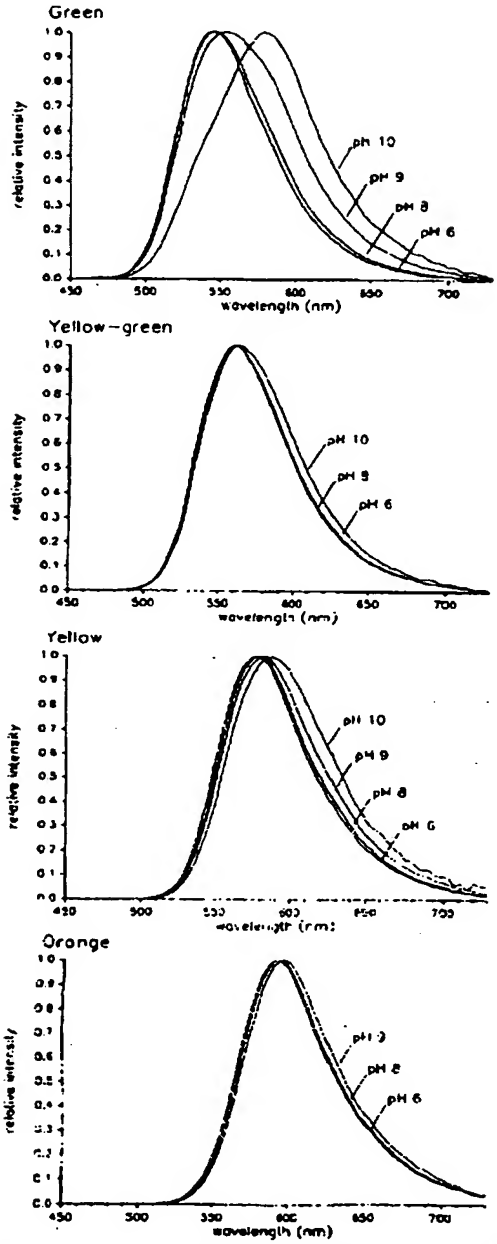

Figure 6. Spectra measured from partially purified lysates of *E. coli* expressing the click beetle luciferases. The intensity maximum for each spectrum has been normalized. The colour emitted by each luciferase at neutral pH is indicated in the corner of each plot. Spectra shown for pH 6.0 and 8.0 were measured in MES/MOPS/Tricine buffer. Spectra shown for pH 9.0 and 10.0 were measured in Tricine/CHES buffer. At pH 8.0, the spectrum measured in MES/MOPS/Tricine was identical to that measured in Tricine/CHES

same rate. The decay rate of these two components is the same rate in the spectrum of the native luciferase. The spectra of the luciferase produced in *E. coli* are of samples that are only partially purified by the method stated above. It can be shown that the differences between this and the native luciferase are not due to intrinsic differences in the enzymes themselves, but instead arise from the effects of the other components in the bacterial lysate. If the native luciferase is mixed with a lysate prepared from *E. coli* which does not contain a luciferase cDNA clone, the spectrum of the mixture is the same as that of lysates containing the luciferase produced in *E. coli*.

These observations reveal two aspects of the effects of an *E. coli* lysate on the spectrum of firefly luciferase. One is that the lysate contains a component that causes luciferase to resist the effects of pH on its spectrum. The other feature is

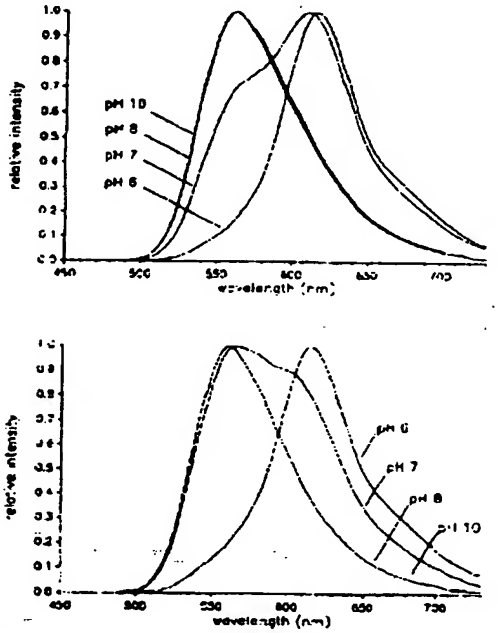

Figure 7. Spectra measured from purified native firefly luciferase (upper plot) and from partially purified lysates of *E. coli* expressing the firefly luciferase (lower plot). The intensity maximum for each spectrum has been normalized. Spectra shown for pH 6.0 and 7.0 were measured in MES/MOPS/Tricine buffer. Spectra shown for pH 8.0 and 10.0 were measured in Tricine/CHES buffer. At pH 8.0, the spectrum measured in MES/MOPS/Tricine had a slightly greater contribution from the red component than that measured in Tricine/CHES

that firefly luciferase, in the presence of the lysate, is in at least two different forms distinguished both by the colour of light emitted, and by different decay rates of the light output. These two features may indicate a single phenomena. That is, a factor in the lysates may be stabilizing some of the luciferase molecules both to destabilizing effects of low pH, and to the temporal loss of enzymatic activity. The presence of the bacterial lysate does not appear to affect the spectral distribution of each of the components of the firefly luciferase spectrum, just their relative contribution to the total spectrum. While this effect is most evident when the spectrum is measured at pH 7.0, it is also evident at pHs 6.0 and 8.0. In these cases, however, the differences are slight since the spectrum consists almost entirely of a single component. Extrapolation of these results to the spectra of the click beetle luciferases incidate that their spectral distributions in the pH range of 6 to 8 are probably not affected by the lysate. This is true since the spectrum of these luciferases is apparently a single component in this pH range. But the pH required to shift the spectra to longer wavelength is potentially different than what would be expected for purified enzymes. However, the spectra of the green-emitting click beetle luciferase at pH 9.0 or 10.0, which also consists of two components, did not reveal the nonuniform decay rate evident with the firefly luciferase at pH 7.0.

SEQUENCE COMPARISON OF CLICK BEETLE AND FIREFLY LUCIFERASES

Our inability to demonstrate cross-hybridization of their corresponding nucleic acid in Southern blots suggested that a significant degree of evolutionary divergence had occurred between the firefly and click beetle luciferases. Sequence analysis of the click beetle cDNA clones has confirmed this. For a direct comparison with the firefly luciferase, the yellow-green-emitting click beetle luciferase was used since its spectral maximum is at nearly the same wavelength. The cDNA encoding this luciferase contains an open reading frame corresponding to 543 amino acids. This is seven amino acids less than that found with the firefly luciferase cDNA. Alignment of the amino acid sequences, deduced from the cDNA sequences, reveals a 47% identity between the two luciferases (Fig. 8). The difference in the number of amino acids between the sequences is mostly accounted for by six gaps in the sequence alignment. These gaps are small, being one or two amino acid in length and, for some, their exact position is somewhat arbitrary.

Throughout the alignment there are no regions of especially high sequence similarity. Thus there is no indication of which regions may have been conserved owing to catalytic or structural constraints on the enzymes. An exception to this is in the last three amino acids which, for the firefly luciferase, have been shown to be necessary for translocation into peroxisomes (Gould and Subramani, 1988b, 1988c). Given the close functional similarity of these enzymes, it is almost certain that the click beetle luciferases are also located in peroxisomes. The hydropathy plots of firefly and click beetle luciferase shown some similarities, but overall appear to be quite different (Fig. 9) (Kyte and Doolittle, 1982). The most apparent similarity is the three large hydrophobic regions found in both luciferases. Other regions of hydrophobicity and hydrophilicity can be found in common between the luciferases, but they are largely obscured in as many differences.

In contrast to the low degree of similarity between the firefly and click beetle luciferases, the similarity between the various click beetle luciferases is very high. Between the luciferases which are capable of emitting different colours, the amino acid sequences are from 95% to 99% identical (Wood *et al.*, 1988b). Since the only difference between these luciferases are the amino acid sequences, the determinants of colour must be found in the relatively few differences between the sequences. We have begun to examine exactly which of the amino acids can affect the colour of light, and have found that not all of the differences between the clones are effective. In some cases, the amino acid determinants of colour may be as few as two or three. This work will be presented elsewhere.

USE OF CLICK BEETLE LUCIFERASES AS GENETIC REPORTERS

It appears that the click beetle luciferases will have all the advantages of the firefly luciferase in their application as reporters of genetic activity. Advantages such as the sensitivity with which they can be detected, or the ability to detect them

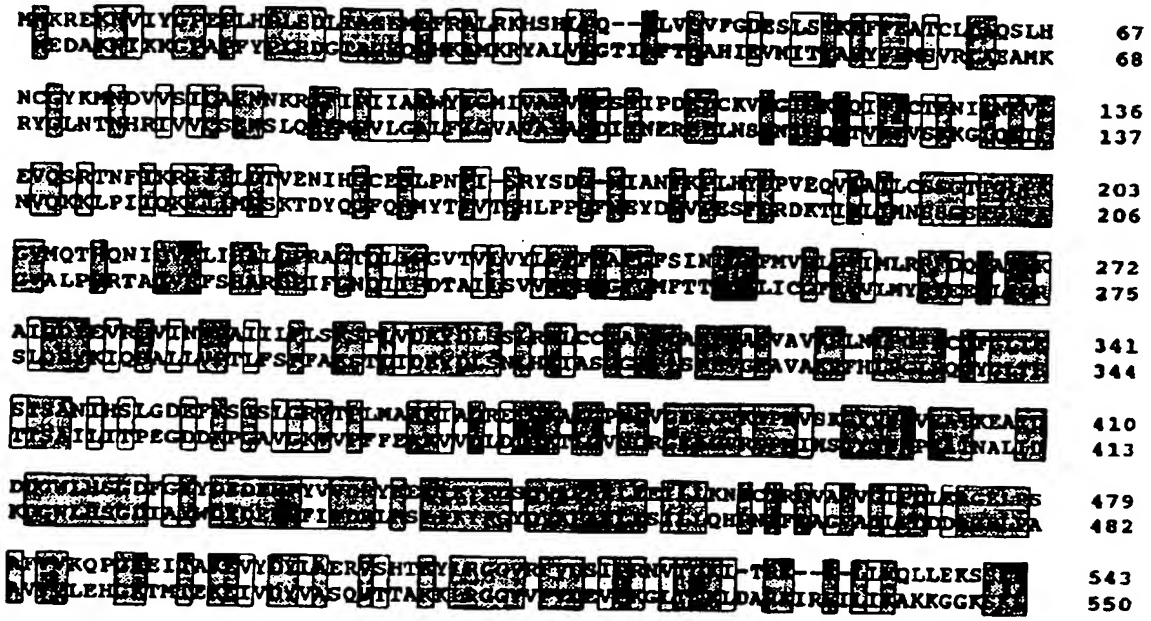

Figure 8. Alignment of the yellow-green-emitting click beetle luciferase (top line) and the firefly luciferase (bottom line). Regions where the aligned amino acids are identical are indicated by dark grey boxes; regions where the amino acids are similar, but not identical, are indicated by light grey boxes. Gaps in the alignment are indicated by hyphens. Numbers on the right indicate the position of the amino acid at the end of each line

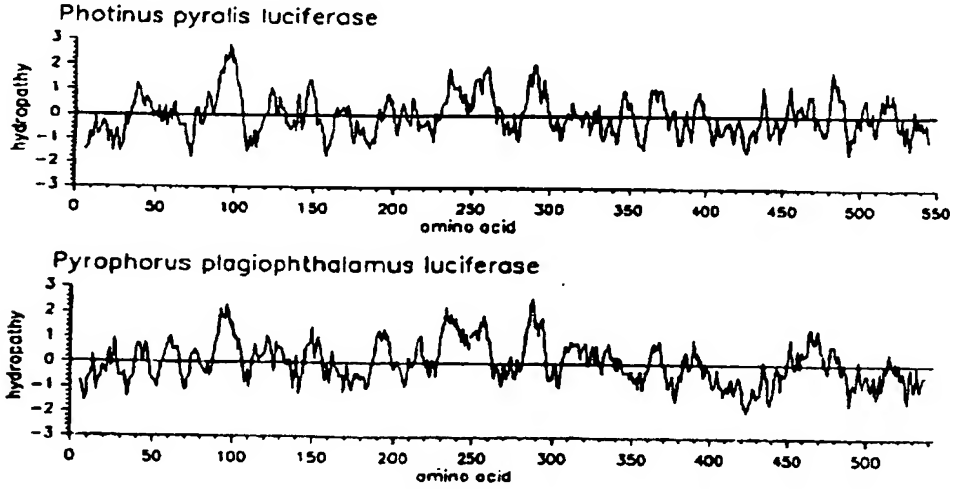

Figure 9. Hydropathy plot of the click beetle (lower plot) and firefly (upper plot) luciferases. Plots were calculated by the Kyte–Doolittle method with a window of 11

in living cells, are evident. Other features of the luciferases, such as their specific activity, or the linearity of their assay with respect to enzyme concentration, have yet to be established. We are at present improving the expression of these luciferases in *E. coli* to provide a source of enzyme for better characterization. It may be expected with the large difference in sequence between the click beetle and firefly luciferases, that these luciferases also have significant differences in their chemical properties as well. This is supported by the dramatic difference in the response of their spectra to changes in pH. By qualitative observation, temperatures above 40 °C or the presence of Zn^{2+} do not cause changes in the spectra of the click beetle luciferases. As noted above, these conditions will cause the firefly luciferase to emit red light. In fact, the temperataure optimum for the click beetle luciferases may be higher than for the firefly luciferase. Other initial experiments suggest that the click beetle luciferases may be more resistant to denaturation by charged detergents, or activation by neutral detergents (Kricka and DeLuca, 1982). Collectively, these observations suggest that the activity of the click beetle luciferases may be less sensitive to environmental conditions. However, these conclusions are tentative since they were made from luciferases in the presence of other components of the *E. coli* lysate.

A novel feature of the click beetle clones is the ability to distinguish between them by the colour of light emitted. This may make them particularly useful as genetic reporters where multiple reporters are desirable. Because their respective sequences differ by only a few amino acids, characteristics of their expression in exogenous hosts should also differ little. The differences in the colour of light would normally have no effect on the hosts, but regardless, expression of a luciferase reporter gene is generally done in the absence of the luciferin substrate. Thus there is no luminescent activity until the actual time of the luciferase assay. The spectral distribution of the luciferases are rather broad which would limit the ability to distinguish each luciferase in a mixture if their respective amounts vary widely. The greatest distinction can be made between the green and orange-emitting clones, which should be distinguishable in a luminometer with the use of optical cut-off filters. From calculations based on the overlap of their spectra alone, and assuming a coefficient of variation of 4% in the assay of luminescence, this method should allow the detection of one of the luciferases in the presence of a 25-fold excess of the other. Since the colours of these luciferases are not easily altered by pH or temperature, it should be possible to distinguish these luciferases *in vivo* as well as *in vitro*. This type of dual reporter gene system would allow one to monitor different promoters within a single host, or to follow different populations of cells simultaneously, each labelled with a different luciferase. The structural similarity of the luciferases increases confidence that differential effects noted in an experiment are properties of the system being observed, and are not artefacts due to individual peculiarities of the reporter genes themselves.

SUMMARY

Firefly luciferase has been used as a tool of scientific investigation for over two decades because of the high sensitivity with which its enzymatic activity can be assayed. With the advent of techniques in nucleic acid manipulations, it has found its newest area of application as a reporter of genetic activity within living cells. In addition to high sensitivity, its assay is rapid and does not require complex procedures or precautions. In comparison to the CAT assay, firefly luciferase has been shown to be well suited as a genetic reporter. But, whereas previously firefly luciferase was the epitome of beetle luciferases because of its availability, cloning techniques have made feasible the study of other luciferases of this type. Some of these luciferases may have additional features enhancing their use as reporters, or in other applications. Our recent cloning of several luciferases from a bioluminescent click beetle substantiates this possibility. These luciferases are unique in the ability to produce bioluminescence of several different colours. In addition, the sequence of these luciferases is considerably different from that of the firefly luciferase, suggesting that other chemical properties of these enzymes will be different. One area where such differences are apparent is in the response of the bioluminescence spectra to changes in pH. We are currently investigating other properties of these new luciferases to better understand their general nature and to determine their suitability in applications.

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(57) Abstract

The invention provides active, non-naturally occurring mutants of beetle luciferases and DNAs which encode such mutants. A mutant luciferase of the invention differs from the corresponding wild-type luciferase by producing bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from the wavelength of peak intensity of the bioluminescence produced by the wild-type enzyme. The mutant luciferases and DNAs of the invention are employed in various biosensing applications.

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MUTANT LUCIFERASES

TECHNICAL FIELD

This invention generally relates to luciferase enzymes that produce luminescence, like that from fireflies. More particularly, the invention concerns mutant luciferases of beetles. The mutant luciferases of the invention are made by genetic engineering, do not occur in nature, and, in each case, include modifications which cause a change in color in the luminescence that is produced. The luciferases of the invention can be used, like their naturally occurring counterparts, to provide luminescent signals in tests or assays for various substances or phenomena.

BACKGROUND OF THE INVENTION

The use of reporter molecules or labels to qualitatively or quantitatively monitor molecular events is well established. They are found in assays for medical diagnosis, for the detection of toxins and other substances in industrial environments, and for basic and applied research in biology, biomedicine, and biochemistry. Such assays include immunoassays, nucleic acid probe hybridization assays, and assays in which a reporter enzyme or other protein is produced by expression under control of a particular promoter. Reporter molecules, or labels in such assay systems, have included radioactive isotopes, fluorescent agents, enzymes and chemiluminescent agents.

Included in the assay system employing chemiluminescence to monitor or measure events of interest are assays which measure the activity of a bioluminescent enzyme, luciferase.

Light-emitting systems have been known and isolated from many luminescent organisms including bacteria, protozoa, coelenterates, molluscs, fish, millipedes, flies, fungi, worms, crustaceans, and beetles, particularly click beetles of genus *Pyrophorus* and the fireflies of the genera *Photinus*, *Photuris*, and

Luciola. In many of these organisms, enzymes catalyze monooxygenations and utilize the resulting free energy to excite a molecule to a high energy state. Visible light is emitted when the excited molecule spontaneously returns to the ground state. This emitted light is called "bioluminescence." Hereinafter it may also be referred to simply as "luminescence."

The limited occurrence of natural bioluminescence is an advantage of using luciferase enzymes as reporter groups to monitor molecular events. Because natural bioluminescence is so rare, it is unlikely that light production from other biological processes will obscure the activity of a luciferase introduced into a biological system. Therefore, even in a complex environment, light detection will provide a clear indication of luciferase activity.

Luciferases possess additional features which render them particularly useful as reporter molecules for biosensing (using a reporter system to reveal properties of a biological system). Signal transduction in biosensors (sensors which comprise a bilogical component) generally involves a two step process: signal generation through a biological component, and signal transduction and amplification through an electrical component. Signal generation is typically achieved through binding or catalysis. Conversion of these biochemical events into an electrical signal is typically based on electrochemical or caloric detection methods, which are limited by the free energy change of the biochemical reactions. For most reactions this is less than the energy of hydrolysis for two molecules of ATP, or about 70 kJ/mole. However, the luminescence elicited by luciferases carries a much higher energy content. Photons emitted from the reaction catalyzed by firefly luciferase (560 nm) have 214 Kj/einstein. Furthermore, the reaction catalyzed by luciferase is one of the most efficient bioluminescent reactions known, having a

quantum yield of nearly 0.9. This enzyme is therefore an extremely efficient transducer of chemical energy.

Since the earliest studies, beetle luciferases, particularly that from the common North American firefly species *Photinus pyralis*, have served as paradigms for understanding of bioluminescence . The fundamental knowledge and applications of luciferase have been based on a single enzyme, called "firefly luciferase," derived from *Photinus pyralis*. However, there are roughly 1800 species of luminous beetles worldwide. Thus, the luciferase of Photinus pyralis is a single example of a large and diverse group of beetle luciferases. It is known that all beetle luciferases catalyze a reaction of the same substrate, a polyheterocyclic organic acid, D-(-)-2-(6'-hydroxy-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (hereinafter referred to as "luciferin", unless otherwise indicated), which is converted to a high energy molecule. It is likely that the catalyzed reaction entails the same mechanism in each case.

The general scheme involved in the mechanism of beetle bioluminescence appears to be one by which the production of light takes place after the oxidative decarboxylation of the luciferin, through interaction of the oxidized luciferin with the enzyme. The color of the light apparently is determined by the spatial organization of the enzyme's amino acids which interact with the oxidized luciferin.

The luciferase-catalyzed reaction which yields bioluminescence (hereinafter referred to simply as "the luciferase-luciferin reaction") has been described as a two-step process involving luciferin, adenosine triphosphate (ATP), and molecular oxygen. In the initial reaction, the luciferin and ATP react to form luciferyl adenylate with the elimination of inorganic pyrophosphate, as indicated in the following reaction:

$$E + LH_2 + ATP \rightleftharpoons E \cdot LH\text{-}AMP + PP_i$$

where E is the luciferase, LH_2 is luciferin, and PP_i is pyrophosphate. The luciferyl adenylate, LH_2-AMP, remains tightly bound to the catalytic site of luciferase. When this form of the enzyme is exposed to molecular oxygen, the enzyme-bound luciferyl adenylate is oxidized to yield oxyluciferin (L=O) in an electronically excited state. The excited oxidized luciferin emits light on returning to the ground state as indicated in the following reaction:

$$E\text{-}LH_2\text{-}AMP + O_2 \Rightarrow (E \cdot L{=}O \cdot AMP)^* + 2H^+ + CO_2$$

$$\downarrow$$

$$E \cdot L = O \cdot AMP + light$$

One quantum of light is emitted for each molecule of luciferin oxidized. The electronically excited state of the oxidized luciferin is a characteristic state of the luciferase-luciferin reaction of a beetle luciferase; the color (and, therefore, the energy) of the light emitted upon return of the oxidized luciferin to the ground state is determined by the enzyme, as evidenced by the fact that various species of beetles having the same luciferin emit differently colored light.

Luciferases have been isolated directly from various sources. The cDNAs encoding luciferases of various beetle species have been reported. (See de Wet et al., Molec. Cell. Biol 7, 725 - 737 (1987); Masuda et al., Gene 77, 265 - 270 (1989); Wood et al., Science 244, 700 - 702 (1989)). With the cDNA encoding a beetle luciferase in hand, it is entirely straightforward for the skilled to prepare large amounts of the luciferase by isolation from bacteria (e.g., E. coli), yeast, mammalian cells in culture, or the like, which have been transformed to express the cDNA. Alternatively, the cDNA, under control of an appropriate promoter and other

signals for controlling expression, can be used in such a cell to provide luciferase, and ultimately bioluminescence catalyzed thereby, as a signal to indicate activity of the promoter. The activity of the promoter may, in turn, reflect another factor that is sought to be monitored, such as the concentration of a substance that induces or represses the activity of the promoter. Various cell-free systems, that have recently become available to make proteins from nucleic acids encoding them, can also be used to make beetle luciferases.

Further, the availability of cDNAS encoding beetle luciferases and the ability to rapidly screen for cDNAS that encode enzymes which catalyze the luciferase-luciferin reaction (see de Wet et al., supra and Wood et al., supra) also allow the skilled to prepare, and obtain in large amounts, other luciferases that retain activity in catalyzing production of bioluminescence through the luciferase-luciferin reaction. These other luciferases can also be prepared, and the cDNAs that encode them can also be used, as indicated in the previous paragraph. In the present disclosure, the term "beetle luciferase" or "luciferase" means an enzyme that is capable of catalyzing the oxidation of luciferin to yield bioluminescence, as outlined above.

The ready availability of cDNAS encoding beetle luciferases makes possible the use of the luciferases as reporters in assays employed to signal, monitor or measure genetic events associated with transcription and translation, by coupling expression of such a cDNA, and consequently production of the enzyme, to such genetic events.

Firefly luciferase has been widely used to detect promoter activity in eucaryotes. Though this enzyme has also been used in procaryotes, the utility of firefly luciferase as genetic reporter in bacteria is not commonly recognized. As genetic reporters, beetle

luciferases are particularly useful since they are monomeric products of a single gene. In addition, no post-translational modifications are required for enzymatic activity, and the enzyme contains no prosthetic groups, bound cofactors, or disulfide bonds. Luminescence from E.coli containing the gene for firefly luciferase can be triggered by adding the substrate luciferin to the growth medium. Luciferin readily penetrates biological membranes and cannot be used as a carbon or nitrogen source by E.coli. The other substrates required for the bioluminescent reaction, oxygen and ATP, are available within living cells. However, measurable variations in luminescence color from luciferases would be needed for systems which utilize two or more different luciferases as reporters (signal geneators).

Clones of different beetle luciferases, particularly of a single genus or species, can be utilized together in bioluminescent reporter systems. Expression in exogenous hosts should differ little between these luciferases because of their close sequence similarity. Thus, in particular, the click beetle luciferases may provide a multiple reporter system that can allow the activity of two or more different promoters to be monitored within a single host, or for different populations of cells to be observed simultaneously. The ability to distinguish each of the luciferases in a mixture, however, is limited by the width of their emissions spectra.

One of the most spectacular examples of luminescence color variation occurs in *Pyrophorus plagiophthalamus*, a large click beetle indigenous to the Caribbean. This beetle has two sets of light organs, a pair on the dorsal surface of the prothorax, and a single organ in a ventral cleft of the abdomen. Four different luciferase clones have been isolated from the ventral organ. The luciferin-luciferase reactions catalyzed by

these enzymes produces light that ranges from green to orange.

Spectral data from the luciferase-luciferin reaction catalyzed by these four luciferases show four overlapping peaks of nearly even spacing, emitting green (peak intensity: 546 nanometers), yellow-green (peak intensity: 560 nanometers), yellow (peak intensity: 578 nanometers) and orange (peak intensity: 593 nanometers) light. The respective proteins are named Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE and Luc*Ppl*OR. Though the wavelengths of peak intensity of the light emitted by these luciferases range over nearly 50 nm, there is still considerable overlap among the spectra, even those with peaks at 546 and 593 nm. Increasing the difference in wavelength of peak intensity would thus be useful to obtain greater measurement precision in systems using two or more luciferases.

The amino acid sequences of the four luciferases from the ventral organ are highly similar. Comparisons of the sequences show them to be 95 to 99% identical.

It would be desirable to enhance the utility of beetle luciferases for use in systems using multiple reporters to effect mutations in luciferase-encoding cDNAs to produce mutant luciferases which, in the luciferase-luciferin reaction, produce light with differences between wavelengths of peak intensity that are greater than those available using currently available luciferases.

Beetle luciferases are particularly suited for producing these mutant luciferases since color variation is a direct result of changes in the amino acid sequence.

Mutant luciferases of fireflies of genus *Luciola* are known in the art. Kajiyama et al., U.S. Patent Nos. 5,219,737 and 5,229,285.

In using luciferase expression in eukaryotic cells for biosensing, it would be desirable to reduce transport of the luciferase to peroxisomes. Sommer et

al., Mol. Biol. Cell *3*, 749 - 759 (1992), have described mutations in the three carboxy-terminal amino acids of P. pyralis luciferase that significantly reduce peroxisome-targeting of the enzyme.

The sequences of cDNAs enoding various beetle luciferases, and the amino acid sequences deduced from the cDNA sequences, are known, as indicated in Table I.

Table I

References for cDNA and Amino Acid Sequences of Various Wild-Type Beetle Luciferases

Luciferase	Reference
Luc*Ppl*GR	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989), see also SEQ ID NO:1; Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*YG	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*YE	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*OR	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Photinus pyralis	de Wet *et al.*, Mol. Cell. Biol. *7*, 725 - 737 (1987); K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700 - 702 (1989)
Luciola cruciata	Kajiyama *et al.*, United States Patent No. 5,229,285; Masuda *et al.*, United States Patent No. 4,968,613
Luciola lateralis	Kajiyama *et al.*, United States Patent No. 5,229,285
Luciola mingrelica	Devine *et al.*, Biochim. et Biophys. Acta *1173*, 121-132(1993)

The cDNA and amino acid sequences of Luc*Ppl*GR, the green-emitting luciferase of the elaterid beetle *Pyrophorus plagiophthalamus*, are shown in SEQ ID NO:1.

SUMMARY OF THE INVENTION

The present invention provides mutant luciferases of beetles and DNAs which encode the mutant luciferases. Preferably, the mutant luciferases produce a light of different color from that of the corresponding wild-type luciferase and preferably this difference in color is such that the wavelength of peak intensity of the luminescence of the mutant differs by at least 1 nm from that of the wild-type enzyme.

The mutant luciferases of the invention differ from the corresponding wild-type enzymes by one or more, but typically fewer than three, amino acid substitutions. The luciferases of the invention may also entail changes in one or more of the three carboxy-terminal amino acids to reduce peroxisome targeting.

In one surprising aspect of the invention, it has been discovered that combining in a single mutant two amino acid substitions, each of which, by itself, occasions a change in color (shift in wavelength of peak intensity) of bioluminescence, causes the mutant to have a shift in wavelength of peak intensity that is greater than either shift caused by the single amino acid substitutions.

cDNAs encoding the mutant luciferases of the invention may be obtained straightforwardly by any standard, site-directed mutagenesis procedure carried out with a cDNA encoding the corresponding wild-type enzyme or another mutant. The mutant luciferases of the invention can be made by standard procedures for expressing the cDNAs which encode them in prokaryotic or eukaryotic cells.

A fuller appreciation of the invention will be gained upon examination of the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

In the following description and examples, process steps are carried out and concentrations are measured at room temperature (about 20 °C to 25 °C) and atmospheric pressure unless otherwise specified.

All amino acids referred to in the specification, except the non-enantiomorphic glycine, are L-amino acids unless specified otherwise. An amino acid may be referred to using the one-letter or three-letter designation, as indicated in the following Table II.

Table II

Designations for Amino Acids

Amino Acid	Three-Letter Designation	One-Letter Designation
L-alanine	Ala	A
L-arginine	Arg	R
L-asparagine	Asn	N
L-aspartic acid	Asp	D
L-cysteine	Cys	C
L-glutamic acid	Glu	E
L-glutamine	Gln	Q
glycine	Gly	G
L-histidine	His	H
L-isoleucine	Ile	I
L-leucine	Leu	L
L-lysine	Lys	K
L-methionine	Met	M
L-phenylalanine	Phe	F
L-proline	Pro	P
L-serine	Ser	S
L-threonine	Thr	T
L-tryptophan	Trp	W
L-tyrosine	Tyr	Y
L-valine	Val	V

"X" means any one of the twenty amino acids listed in Table II.

Peptide or polypeptide sequences are written and numbered from the initiating methionine, which is numbered "1," to the carboxy-terminal amino acid.

A substitution at a position in a polypeptide is indicated with [designation for original amino acid]$_{[position\ number]}$[designation for replacing amino acid]. For example, substitution of an alanine at position 100 in a polypeptide with a glutamic acid would be indicated by $Ala_{100}Glu$ or $A_{100}E$. Typically, the substitution will be preceded by a designation for the polypeptide in which the substitution occurs. For example, if the substitution $A_{100}E$ occurs in an hypothetical protein designated "Luck," the substitution would be indicated as Luck-$Ala_{100}Glu$ or Luck-$A_{100}E$. If there is more than one substitution in a polypeptide, the indications of the substitutions are separated by slashes. For example, if the hypothetical protein "Luck" has a substitution of glutamic acid for alanine at position 100 and a substitution of asparagine for lysine at position 150, the polypeptide with the substitutions would be indicated as Luck-$Ala_{100}Glu/Lys_{150}Asn$ or Luck-$A_{100}E/K_{150}N$. To indicate different substitutions at a position in a polypeptide, the designations for the substituting amino acids are separated by commas. For example, if the hypothetical "Luck" has substitutions of glutamic acid, glycine or lysine for alanine at position 100, the designation would be Luck-Ala_{100}/Glu,Gly,Lys or Luck-A_{100}/E,G,K.

The standard, one-letter codes "A," "C," "G," and "T" are used herein for the nucleotides adenylate, cytidylate, guanylate, and thymidylate, respectively. The skilled will understand that, in DNAs, the nucleotides are 2'-deoxyribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) while, in RNAs, the nucleotides are ribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) and uridylate (U) occurs in place of T. "N" means any one of the four nucleotides.

Oligonucleotide or polynucleotide sequences are written from the 5'-end to the 3'-end.

The term "mutant luciferase" is used herein to refer to a luciferase which is not naturally occurring

and has an amino acid sequence that differs from those of naturally occurring luciferases.

In one of its aspects, the present invention is a mutant beetle luciferase which produces bioluminescence (i.e., catalyzes the oxidation of luciferin to produce bioluminescence) which has a shift in wavelength of peak intensity of at least 1 nm from the wavelength of peak intensity of the bioluminescence produced by the corresponding wild-type luciferase and has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided that the mutant optionally has a peroxisome-targeting-avoiding sequence at its carboxy-terminus.

Exemplary mutant luciferases of the invention are those of the group consisting of Luc*Ppl*GR-$R_{215}H$, -$R_{215}G$, -$R_{215}T$, -$R_{215}M$, -$R_{215}P$, -$R_{215}A$, -$R_{215}L$, -$R_{223}L$, -$R_{223}Q$, -$R_{223}M$, -$R_{223}H$, -$V_{224}I$, -$V_{224}S$, -$V_{224}F$, -$V_{224}Y$, -$V_{224}L$, -$V_{224}H$, -$V_{224}G$, -$V_{232}E$, -$V_{236}H$, -$V_{236}W$, -$Y_{237}S$, -$Y_{237}C$, -$L_{238}R$, -$L_{238}M$, -$L_{238}Q$, -$L_{238}S$, -$L_{238}D$, -$H_{242}A$, -$F_{244}L$, -$G_{245}S$, -$G_{245}E$, -$S_{247}H$, -$S_{247}T$, -$S_{247}Y$, -$S_{247}F$, -$I_{248}R$, -$I_{248}V$, -$I_{248}F$, -$I_{248}T$, -$I_{248}S$, -$I_{248}N$, -$H_{348}N$, -$H_{348}Q$, -$H_{348}E$, -$H_{348}C$, -$S_{247}F/F_{246}L$, -$S_{247}F/I_{248}C$, -$S_{247}F/I_{248}T$, -$V_{224}F/R_{215}G$, -$V_{224}F/R_{215}T$, -$V_{224}F/R_{215}V$, -$V_{224}F/R_{215}P$, -$V_{224}F/P_{222}S$, -$V_{224}F/Q_{227}E$, -$V_{224}F/L_{238}V$, -$V_{224}F/L_{238}T$, -$V_{224}F/S_{247}G$, -$V_{224}F/S_{247}H$, -$V_{224}F/S_{247}T$, and -$V_{224}F/S_{247}F$.

The following Table III shows spectral properties of these and other exemplary mutant luciferases.

TABLE III

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
w.t.	545	0	72
$V_{214}S$	*		
Q	*		
Y	*		
K	*		
L	*		
G	*		
C	*		
E	*		
F	*		
P	*		
H	*		
R	*		
$R_{215}H$	562	17	82
Q	567	22	81
G	576	31	82
T	576	31	84
M	582	37	83
P	588	43	91
S	*		
Y	*		
K	*		
L	*		
C	*		
E	*		
F	*		
$R_{223}L$	549	4	75
Q	549	4	73

*Spectral shift (≥ 2 nm) observed by eye.

TABLE III, cont.

Protein	Spectral Shift		
Luc*Ppl*GR-	peak	shift	width
$R_{223}M$	549	4	75
M	551	6	75
S	*		
Y	*		
K	*		
G	*		
C	*		
E	*		
F	*		
P	*		
$V_{224}I$	546	1	75
●	556	11	70
F	561	16	84
Y	565	20	87
L	578	33	94
H	584	39	69
G	584	39	70
$V_{232}E$	554	9	83
$V_{236}H$	554	8	74
W	554	9	74
$Y_{237}S$	553	8	73
C	554	9	74
$L_{238}R$	544	-1	72
M	555	10	75
Q	557	12	76
S	559	14	73
D	568	23	76
$H_{242}A$	559	14	75

*Spectral shift (≥ 2 nm) observed by eye.

TABLE III, cont.

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
$H_{242}S$	561	16	74
$F_{244}L$	555	10	79
$G_{245}S$	558	13	79
E	574	29	79
$S_{247}H$	564	19	72
Y	566	21	79
F	569	24	84
$I_{248}R$	544	-1	72
V	546	1	72
F	548	3	74
T	554	9	75
S	558	13	80
N	577	32	90
$H_{348}A$	592	47	67
C	593	48	66
N	597	52	67
N	605	60	72
$V_{214}C/V_{224}A$	558	14	72
$S_{247}F/F_{246}L$	567	22	79
$S_{247}F/I_{248}C$	586	41	84
$S_{247}F/I_{248}T$	596	51	80
$T_{233}A/L_{238}M$	555	10	75
$V_{282}I/I_{283}V$	563	3	73
$V_{224}F/R_{215}G$	584	39	80
$V_{224}F/R_{215}T$	587	42	80
$V_{224}F/R_{215}V$	589	44	80
$V_{224}F/R_{215}P$	597	52	81
$V_{224}F/P_{222}S$	564	3	86

TABLE III, cont.

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
$V_{224}F/Q_{227}E$	583	38	85
$V_{224}F/L_{238}V$	575	30	85
$V_{224}F/L_{238}M$	576	31	87
$V_{224}F/S_{247}G$	581	36	84
$V_{224}F/S_{247}H$	581	36	79
$V_{224}F/S_{247}Y$	595	50	88
$V_{224}F/S_{247}F$	597	52	85

"Corresponding positions" in luciferases other than Luc*Ppl*GR can be determined either from alignments at the amino acid level that are already known in the art (see, *e.g.*, Wood *et al.*, Science *244*, 700 - 702 (1989); Devine *et al.*, Biochim. et Biophys. Acta *1173*, 121-132(1993)) or by simply aligning at the amino acid level to maximize alignment of identical or conservatively substituted residues, and keeping in mind in particular that amino acids 195 - 205 in the Luc*Ppl*GR sequence are very highly conserved in all beetle luciferases and that there are no gaps for more than 300 positions after that highly conserved 11-mer in any beetle luciferase aminio acid sequence.

A "peroxisome-targeting-avoiding sequence at its carboxy-terminus" means (1) the three carboxy-terminal amino acids of the corresponding wild-type luciferase are entirely missing from the mutant; or (2) the three carboxy-terminal amino acids of the corresponding wild-type luciferase are replaced with a sequence, of one, two or three amino acids that, in accordance with Sommer et al., supra, will reduce peroxisome-targeting by at least 50 %. If the three carboxy-terminal amino acids of the wild-type luciferase are replaced by a three-amino-acid peroxisome-targeting-avoiding sequence in the mutant, and if the sequence in the mutant is $X_1X_2X_3$, where X_3 is carboxy-terminal, than X_1 is any of the twenty amino acids except A, C, G, H, N, P, Q, T and S, X_2 is any of the twenty amino acids except H, M, N, Q, R, S and K, and X_3 is any of the twenty amino acids except I, M, Y and L. Further, any one or two, or all three, of X_1, X_2, and X_3 could be absent from the mutant (i.e., no amino acid corresponding to the position). The most preferred peroxisome-targeting-avoiding sequence is IAV, where V is at the carboxy-terminus.

In another of its aspects, the invention entails a combination of luciferases, in a cell (eukaryotic or prokaryotic), a solution (free or linked as a reporter to

an antibody, antibody-fragment, nucleic acid probe, or the like), or adhererd to a solid surface, optionally through an antibody, antibody fragment or nucleic acid, and exposed to a solution, provided that at least one of the luciferases is a mutant, both of the luciferases remain active in producing bioluminescence, and the wavelengths of peak intensities of the bioluminescence of the luciferases differ because the amino acid sequences of the luciferases differ at at least one of the positions corresponding to positions 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348 in the amino acid sequence of Luc*Ppl*GR, provided that one or both of the luciferases optionally have peroxisome-targeting-avoiding sequences.

In another of its aspects, the invention entails a DNA molecule, which may be an eukaryotic or prokaryotic expression vector, which comprises a segment which has a sequence which encodes a mutant beetle luciferase of the invention.

Most preferred among the DNAs of the invention are those with segments which encode a preferred mutant luciferase of the invention.

From the description of the invention provided herein, the skilled will recognize many modifications and variations of what has been described that are within the spirit of the invention. It is intended that such modifications and variations also be understood as part of the invetion.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Promega Corporation
(ii) TITLE OF INVENTION: Mutant Luciferases
(iii) NUMBER OF SEQUENCES: 1
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Foley & Lardner
(B) STREET: P. O. Box 1497
(C) CITY: Madison
(D) STATE: Wisconsin
(E) COUNTRY: US
(F) ZIP: 53701-1497
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/177,081
(B) FILING DATE:3-Jan-1994
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Scanlon, William J.
(B) REGISTRATION NUMBER: 30136
(C) REFERENCE/DOCKET NUMBER: 19017/148P
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (608) 258-4284
(B) TELEFAX: (608) 258-4258

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1632 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG ATG AAG AGA GAG AAA AAT GTT GTA TAT GGA CCC GAA CCC CTA CAC     48
Met Met Lys Arg Glu Lys Asn Val Val Tyr Gly Pro Glu Pro Leu His
                5                   10                  15

CCC TTG GAA GAC TTA ACA GCA GGA GAA ATG CTC TTC AGG GCC CTT CGA     96
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
            20                  25                  30

AAA CAT TCT CAT TTA CCG CAG GCT TTA GTA GAT GTG TAT GGT GAA GAA    144
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Tyr Gly Glu Glu
        35                  40                  45

TGG ATT TCA TAT AAA GAG TTT TTT GAA ACT ACA TGC CTA CTA GCA CAA    192
Trp Ile Ser Tyr Lys Glu Phe Phe Glu Thr Thr Cys Leu Leu Ala Gln
    50                  55                  60

AGT CTT CAC AAT TGT GGA TAC AAG ATG AGT GAT GTA GTG TCG ATC TGC    240
Ser Leu His Asn Cys Gly Tyr Lys Met Ser Asp Val Val Ser Ile Cys
65                  70                  75                  80

GCG GAG AAC AAT AAA AGA TTT TTT GTT CCC ATT ATT GCA GCT TGG TAT    288
Ala Glu Asn Asn Lys Arg Phe Phe Val Pro Ile Ile Ala Ala Trp Tyr
                85                  90                  95

ATT GGT ATG ATT GTA GCA CCT GTT AAT GAG GGC TAC ATC CCA GAT GAA    336
Ile Gly Met Ile Val Ala Pro Val Asn Glu Gly Tyr Ile Pro Asp Glu
            100                 105                 110

CTC TGT AAG GTC ATG GGT ATA TCG AGA CCA CAA CTA GTT TTT TGT ACA    384
Leu Cys Lys Val Met Gly Ile Ser Arg Pro Gln Leu Val Phe Cys Thr
        115                 120                 125
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AAG AAT ATT CTA AAT AAG GTA TTG GAG GTA CAG AGC AGA ACT GAT TTC   432
Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asp Phe
    130                 135                 140

ATA AAA AGG ATT ATC ATA CTA GAT GCT GTA GAA AAC ATA CAC GGT TGT   480
Ile Lys Arg Ile Ile Ile Leu Asp Ala Val Glu Asn Ile His Gly Cys
145                 150                 155                 160

GAA AGT CTT CCC AAT TTT ATT TCT CGT TAT TCG GAT GGA AAT ATT GCC   528
Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala
                165                 170                 175

AAC TTC AAA CCT TTA CAT TAC GAT CCT GTT GAA CAA GTG GCA GCT ATC   576
Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile
            180                 185                 190

TTA TGT TCG TCA GGC ACA ACT GGA TTA CCG AAA GGT GTA ATG CAA ACT   624
Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr
        195                 200                 205

CAT AGA AAT GTT TGT GTC CGA CTT ATA CAT GCT TTA GAC CCC AGG GTA   672
His Arg Asn Val Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Val
    210                 215                 220

GGA ACG CAA CTT ATT CCT GGT GTG ACA GTC TTA GTA TAT CTG CCT TTT   720
Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe
225                 230                 235                 240

TTC CAT GCT TTT GGG TTC TCT ATA AAC TTG GGA TAC TTC ATG GTG GGT   768
Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly
                245                 250                 255

CTT CGT GTT ATC ATG TTA AGA CGA TTT GAT CAA GAA GCA TTT CTA AAA   816
Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys
            260                 265                 270

GCT ATT CAG GAT TAT GAA GTT CGA AGT GTA ATT AAC GTT CCA GCA ATA   864
Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile
        275                 280                 285

ATA TTG TTC TTA TCG AAA AGT CCT TTG GTT GAC AAA TAC GAT TTA TCA   912
Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser
    290                 295                 300

AGT TTA AGG GAA TTG TGT TGC GGT GCG GCA CCA TTA GCA AAG GAA GTT   960
Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val
305                 310                 315                 320

GCT GAG ATT GCA GTA AAA CGA TTA AAC TTG CCA GGA ATT CGC TGT GGA  1008
Ala Glu Ile Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly
                325                 330                 335

TTT GGT TTG ACA GAA TCT ACT TCA GCT AAT ATA CAC AGT CTT AGG GAT  1056
Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp
            340                 345                 350

GAA TTT AAA TCA GGA TCA CTT GGA AGA GTT ACT CCT TTA ATG GCA GCT  1104
Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala
        355                 360                 365

AAA ATA GCA GAT AGG GAA ACT GGT AAA GCA TTG GGA CCA AAT CAA GTT  1152
Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val
    370                 375                 380

GGT GAA TTA TGC ATT AAA GGT CCC ATG GTA TCG AAA GGT TAC GTG AAC  1200
Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn
385                 390                 395                 400
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AAT GTA GAA GCT ACC AAA GAA GCT ATT GAT GAT GAT GGT TGG CTT CAC  1248
Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His
                405                 410                 415

TCT GGA GAC TTT GGA TAC TAT GAT GAG GAT GAG CAT TTC TAT GTG GTG  1296
Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val
            420                 425                 430

GAC CGT TAC AAG GAA TTG ATT AAA TAT AAG GGC TCT CAG GTA GCA CCT  1344
Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro
        435                 440                 445

GCA GAA CTA GAA GAG ATT TTA TTG AAA AAT CCA TGT ATC AGA GAT GTT  1392
Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val
    450                 455                 460

GCT GTG GTT GGT ATT CCT GAT CTA GAA GCT GGA GAA CTG CCA TCT GCG  1440
Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala
465                 470                 475                 480

TTT GTG GTT ATA CAG CCC GGA AAG GAG ATT ACA GCT AAA GAA GTT TAC  1488
Phe Val Val Ile Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr
                485                 490                 495

GAT TAT CTT GCC GAG AGG GTC TCC CAT ACA AAG TAT TTG CGT GGA GGG  1536
Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly
            500                 505                 510

GTT CGA TTC GTT GAT AGC ATA CCA AGG AAT GTT ACA GGT AAA ATT ACA  1584
Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr
        515                 520                 525

AGA AAG GAA CTT CTG AAG CAG TTG CTG GAG AAG AGT TCT AAA CTT TAA  1632
Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu
    530                 535                 540
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CLAIMS

1. A mutant beetle luciferase which has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

2. A mutant luciferase according to Claim 1 wherein there is one amino acid substitution.

3. A mutant luciferase according to Claim 1 wherein there are two amino acid substitutions.

4. A mutant luciferase according to Claim 3 wherein each of the amino acid substitutions is at a position corresponding to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

5. A mutant luciferase according to Claim 1 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

6. A mutant luciferase according to Claim 2 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

7. A mutant luciferase according to Claim 3 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

8. A mutant luciferase according to Claim 4 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

9. A mutant luciferase according to Claim 5 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

10. A mutant luciferase according to Claim 6 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

11. A mutant luciferase according to Claim 7 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

12 A mutant luciferase according to Claim 8 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

13. A mutant luciferase of Claim 9 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

14. A mutant luciferase of Claim 10 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

15. A mutant luciferase of Claim 11 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

16. A mutant luciferase of Claim 12 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

17. A mutant luciferase of Claim 13 wherein the mutant is selected from the group consisting of Luc*Ppl*GR-$R_{215}H$, $-R_{215}G$, $-R_{215}T$, $-R_{215}M$, $-R_{215}P$, $-R_{215}A$, $-R_{215}L$, $-R_{223}L$, $-R_{223}Q$, $-R_{223}M$, $-R_{223}H$, $-V_{224}I$, $-V_{224}S$, $-V_{224}F$, $-V_{224}Y$, $-V_{224}L$, $-V_{224}H$, $-V_{224}G$, $-V_{232}E$, $-V_{236}H$, $-V_{236}W$, $-Y_{237}S$, $-Y_{237}C$, $-L_{238}R$, $-L_{238}M$, $-L_{238}Q$, $-L_{238}S$, $-L_{238}D$, $-H_{242}A$, $-F_{244}L$, $-G_{245}S$, $-G_{245}E$, $-S_{247}H$, $-S_{247}T$, $-S_{247}Y$, $-S_{247}F$, $-I_{248}R$, $-I_{248}V$, $-I_{248}F$, $-I_{248}T$, $-I_{248}S$, $-I_{248}N$, $-H_{348}N$, $-H_{348Q}Q$, $-H_{348}E$, $-H_{348}C$, $-S_{247}F/F_{246}L$, $-S_{247}F/I_{248}C$, $-S_{247}F/I_{248}T$, $-V_{224}F/R_{215}G$, $-V_{224}F/R_{215}T$, $-V_{224}F/R_{215}V$, $-V_{224}F/R_{215}P$, $-V_{224}F/P_{222}S$, $-V_{224}F/Q_{227}E$, $-V_{224}F/L_{238}V$, $-V_{224}F/L_{238}T$, $-V_{224}F/S_{247}G$, $-V_{224}F/S_{247}H$, $-V_{224}F/S_{247}T$, and $-V_{224}F/S_{247}F$.

18. A DNA molecule which comprises a segment which has a sequence which encodes a mutant beetle luciferase which has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

19. A DNA molecule according to Claim 18 wherein the encoded mutant luciferase has one amino acid substitution.

20. A DNA molecule according to Claim 18 wherein the encoded mutant luciferase has two amino acid substitutions.

21. A DNA molecule according to Claim 20 wherein, in the encoded mutant luciferase, each of the amino acid substitutions is at a position corresponding to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

22. A DNA molecule according to Claim 18 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

23. A DNA molecule according to Claim 19 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

24. A DNA molecule according to Claim 20 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

25. A DNA molecule according to Claim 21 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

26. A DNA molecule according to Claim 22 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

27. A mutant luciferase according to Claim 23 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

28. A DNA molecule according to Claim 24 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

29. A DNA molecule according to Claim 25 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

30. A DNA molecule according to Claim 26 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

31. A DNA molecule according to Claim 27 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

32. A DNA molecule according to Claim 28 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

33. A DNA molecule according to Claim 29 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

34. A DNA molecule according to Claim 30 wherin the encoded mutant luciferase is selected from the group consisting of Luc*Ppl*GR-$R_{215}H$, -$R_{215}G$, -$R_{215}T$, -$R_{215}M$, -$R_{215}P$, -$R_{215}A$, -$R_{215}L$, -$R_{223}L$, -$R_{223}Q$, -$R_{223}M$, -$R_{223}H$, -$V_{224}I$, -$V_{224}S$, -$V_{224}F$, -$V_{224}Y$, -$V_{224}L$, -$V_{224}H$, -$V_{224}G$, -$V_{232}E$, -$V_{236}H$, -$V_{236}W$, -$Y_{237}S$, -$Y_{237}C$, -$L_{238}R$, -$L_{238}M$, -$L_{238}Q$, -$L_{238}S$, -$L_{238}D$, -$H_{242}A$, -$F_{244}L$, -$G_{245}S$, -$G_{245}E$, -$S_{247}H$, -$S_{247}T$, -$S_{247}Y$, -$S_{247}F$, -$I_{248}R$, -$I_{248}V$, -$I_{248}F$, -$I_{248}T$, -$I_{248}S$, -$I_{248}N$, -$H_{348}N$, -$H_{348Q}Q$, -$H_{348}E$, -$H_{348}C$, -$S_{247}F/F_{246}L$, -$S_{247}F/I_{248}C$, -$S_{247}F/I_{248}T$, -$V_{224}F/R_{215}G$, -$V_{224}F/R_{215}T$, -$V_{224}F/R_{215}V$, -$V_{224}F/R_{215}P$, -$V_{224}F/P_{222}S$, -$V_{224}F/Q_{227}E$, -$V_{224}F/L_{238}V$, -$V_{224}F/L_{238}T$, -$V_{224}F/S_{247}G$, -$V_{224}F/S_{247}H$, -$V_{224}F/S_{247}T$, and -$V_{224}F/S_{247}F$.

INTERNATIONAL SEARCH REPORT

Inter. ..onal application No.
PCT/US95/00108

A. CLASSIFICATION OF SUBJECT MATTER

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US CL :435/189; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/189, 172.3, 320.1, 252.3, 252.33; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS Letters, Volume 307, No. 2, issued July 1992, G. Sala-Newby et al., "Engineering Firefly Luciferase as an Indicator of Cyclic AMP-Dependent Protein Kinase in Living Cells", pages 241-244, see entire document.	1, 2, 5, 6, 18, 19, 22, 23
X	BIOCHEMICAL JOURNAL, Volume 279, issued November 1991, G. Sala-Newby et al., "Engineering a Bioluminescent Indicator for Cyclic AMP-Dependent Protein Kinase", pages 727-732, see entire document.	1, 2, 5, 6, 18, 19, 22, 23
A	PROTEIN ENGINEERING, Volume 4, No. 6, issued August 1991, N. Kajiyama et al., "Isolation and Characterization of Mutants of Firefly Luciferase Which Produce Different Colors of Light", pages 691-693.	1-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 5, issued April 1990, K.V. Wood, "Luc Genes: Introduction of Colour Into Bioluminescence Assays", pages 107-114.	1-34
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 4, issued July 1989, K.V. Wood et al., "Introduction to Beetle Luciferases and Their Applications", pages 289-301.	1-34
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 4, issued July 1989, K.V. Wood et al., "Bioluminescent Click Beetles Revisited", pages 31-39.	1-34
A	SCIENCE, Volume 244, issued 12 May 1989, K.V. Wood et al., "Complementary DNA Coding Click Betle Luciferases Can Elict Bioluminescence of Different Colors", pages 700-702.	1-34

INTERNATIONAL SEARCH REPORT

Inte: ..onal appr...ation No.
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, LIFESCI, EMBASE, WPI, BIOTECHDS, CA
search terms: luciferase#, muta? or modif?, gene# or sequence#, beetle# or firefl?, pyrophorus or plagiophthalamus, photinus or luciola

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(54) Title: LUCIFERASES

(57) Abstract

Proteins are provided having luciferase activity with greater heat stability than wildtype luciferases by replacing the glutamate equivalent to that at position 354 of *Photinus pyralis* luciferase or 356 of *Luciola* luciferases with an alternative amino acid, particularly lysine. DNA, vectors and cells that encode for and express the proteins are also provided as are test kits and reagents for carrying out luminescence assays using the proteins of the invention. Preferred proteins have a second replaced amino acid at a position equivalent to position 215 of *Photinus pyralis* luciferase or 217 of *Luciola* luciferases.

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LUCIFERASES.

The present invention relates to novel proteins having luciferase activity and to DNA and vectors encoding for their expression. Particularly the present invention provides luciferases having heat stability at temperatures above 30°C.

Firefly luciferase catalyses the oxidation of luciferin in the presence of ATP, Mg^{2+} and molecular oxygen with the resultant production of light. This reaction has a quantum yield of about 0.88 (see DeLuca & McElroy (1978) and Seliger & McElroy (1960)) and this light emitting property has led to its use in luminometric assays where ATP levels are being measured.

Luciferase is obtainable directly from the bodies of insects such as fireflies or glow-worms or by expression from microorganisms including recombinant DNA constructs encoding for the enzyme. Four significant species of firefly from which the enzyme may be obtained, or DNA encoding for it may be derived, are the Japanese GENJI and HEIKE fireflies Luciola cruciata and Luciola lateralis, the East European Firefly Luciola mingrelica and the North American firefly (Photinus pyrális). The glow-worm Lampyris noctiluca is a further source with the amino acid sequence of its luciferase having 84% homology to that of Photinus pyralis.

The heat stability of wild and recombinant type luciferases is such that they lose activity quite rapidly when exposed to temperatures in excess of about 30°C, particularly over 35°C. Such instability renders the enzyme deficient when used or stored at high ambient temperatures or if heat induced increase in reaction rate is required. It is known that Japanese firefly luciferase can be stabilised against heat inactivation by mutating it at its position 217 to replace a threonine residue by an isoleucine residue (Kajiyama & Nakano (1993) Biochemistry 32 page 13795 to 13799). In this manner the thermal and

pH stability and the specific activity of the enzyme were increased. The heat stabilisation of Photinus pyralis and Luciola mingrelica luciferases has not yet been reported.

The present inventors have now provided novel luciferases having increased heat stability over wild type luciferases by replacing a glutamate residue present in a sequence conserved in each of Photinus pyralis, Luciola mingrelica, Luciola lateralis and Luciola cruciata with alternative amino acids, particularly lysine or arginine. This glutamate is found at position 354 in Photinus pyralis luciferase, at the third amino acid of the conserved amino acid sequence TPEGDDKPGA found in the luciferases of this and the other species.

Thus in the first aspect of the invention there is provided a protein having luciferase activity and having over 60% homology of amino acid sequence with that of Photinus pyralis, Luciola mingrelica, Luciola cruciata or Luciola lateralis characterised in that the amino acid residue corresponding to residue 354 of Photinus pyralis luciferase and residue 356 of Luciola mingrelica, Luciola cruciata and Luciola lateralis luciferase is an amino acid other than glutamate.

The amino acid may be a naturally occurring amino acid or may be a so called unusual amino acid such as an modified naturally occurring amino acid or an analogue of such. Analogues of amino acids other than glutamate will be understood to be those compounds that have equivalent effect on the protein to the amino acid of which they are analogues. Typical unusual amino acids are those as set out in the US and European Patentin Manuals and the Rules of Practice in Patent Cases: application disclosures containing nucleotide and/or amino acid sequences: modified and unusual amino acids.

Preferably the protein is characterised in that it comprises an amino acid sequence XGDDKPGA wherein X is the amino acid other than glutamate. More preferably the protein comprises the amino acid

sequence TPXGDDKPGA and preferably, for thermostability, X is any amino acid other than aspartic acid, proline or glycine; still more preferably it is tryptophan, valine, leucine, isoleucine or asparagine but most preferably is lysine or arginine, or analogue of any of these.

It will be realised that some species may have luciferases with one or two amino acids different in this conserved TPXGDDKPA region, but all active proteins corresponding to such luciferases that are altered to the extent that the amino acid at position three in the sequence is not glutamate are provided for by the present invention.

In preferred forms of the present invention the protein of the invention also has the amino acid at the position corresponding to amino acid 217 of the Luciola firefly luciferases or 215 of Photinus pyralis changed to a hydrophobic amino acid, preferably to isoleucine, leucine or valine, as described in EP 0524448 A. Such change has been found to result in an increase in thermostability over the 354 change alone; thus the two changes have effects that are substantially independent of each other and which may be used together.

In a second aspect of the invention there is provided DNA encoding for the protein of the invention and in a third aspect there is provided a vector, particularly a plasmid, comprising a *luc* gene (the gene encoding for luciferase) in such a form as to be capable of expressing the protein of the invention. Such forms are those where the vector includes DNA sequences capable of controlling the expression of the protein of the invention such that when incorporated into a microorganism host cell the protein may readily be expressed as required, if necessary by addition of suitable inducers.

The *luc* genes for Photinus pyralis, Luciola mingrelica, Luciola cruciata and Luciola lateralis are all known and isolatable by standard molecular biology techniques. Photinus pyralis *luc* gene is commercially available form Promega as the plasmid pGEM. Thus

convenient methods and sources for deriving starting material for production of DNA of the invention are (i) use of naturally occurring firefly genomic DNA and amplifying the *luc* gene from it using eg, PCR, (ii) pGEM and (iii) pGLf37 plasmid of Kajiyama & Nakano. Further genes encoding for proteins having luciferase activity, ie. the activity of oxidising luciferin with the emission of light, will also be suitable sources for starting material for obtaining a DNA, and ultimately through gene expression, a protein of the invention.

Suitable vectors for use in manipulating wild type or other *luc* gene DNA in order to produce the DNA of the invention will be any vector in which the DNA can be contained within while alteration of the naturally occurring glutamate to an alternative amino acid is carried out. For chemically induced mutagenesis, eg. using agents such as hydroxylamine, this is not particularly critical and many suitable vectors will occur to those skilled in the art that will allow easy manipulation of the gene before and after the mutagenic process.

It may be preferred to specifically mutate the *luc* gene at the glutamate and thus a site directed mutagenesis operation will be required. Such operations may be most easily carried out in vectors and these will be well known to those skilled in the art.

For expression of *luc* genes of wild and known type, and those of the present invention suitable vectors include pKK223-3, pDR540 (available from Boehringer Mannheim) and pT7-7; the first two having the tac promoter under control of the lactose repressor allowing expression to be induced by presence of isopropyl-thiogalactoside (IPTG). pT7-7 allows control by the T7-RNA polymerase promoter and thus provides the basis for a very high level of gene expression in E. coli cells containing T7 RNA polymerase. Of these vectors expression is found to be highest when the *luc* genes are inserted into the pT7-7 vector.

Expression of luciferase from a *luc* gene inserted into pKK223-3 and

pDR540 results in the expression of wild-type N-terminal sequence luciferase whilst expression from a *luc* gene inserted into pT7-7 results in synthesis of a fusion protein with extra N-terminal amino acids M-A-R-I-Q. The ribosome binding site and start codon of the *luc* gene in each of the vectors with the *luc* gene present (named constructs pPW204, pPW116 and pPW304) are shown in Table 1 of the Examples.

A third aspect of the present invention provides cells capable of expressing the proteins of the invention; methods for producing such proteins using these cells and test kits and reagents comprising the proteins of the invention. Also provided are assay methods wherein ATP is measured using luciferin/luciferase reagents, as is well known in the art, characterised in that the luciferase is a protein of the invention. Luciferase preparations of the invention are relatively thermostable at 30-70°C, particularly 37-60°C, and especially 40-50°C as compared to the wild-type and recombinant wild-type luciferases.

Any cell capable of expressing heterologous protein using DNA sequences in its DNA, or in vectors such as plasmids contained in the cell, may be used to express the proteins of the invention. Typical of such cells will be yeast and bacterial cells such as Saccharomyces cerevisiae and Escherichia coli cells, but many other host organisms suitable for the purpose of protein expression will occur to those skilled in the art. Insect cells may be preferred as the protein is an insect protein. The protein may be expressed as a protein of similar structure to native and known recombinant luciferases, or may be expressed as a fusion or conjugate of such proteins with other amino acids, peptides, proteins or other chemical entities, eg. the M-A-R-I-Q sequence above.

It will be realised by those skilled in the art that certain hosts may have particular codon preferences, eg. bacteria in some cases use different codons to yeast, and thus the DNA incorporated into such a host may advantageously be altered to provide a degenerate codon for a given amino acid that will give more favourable expression in that

host. Such degenerate DNAs are of course included in the scope of the DNA of the invention.

E. coli BL21(DE3) is one suitable host and has the T7 RNA polymerase integrated stably into its chromosome under control of the inducible lacUV5 promoter and is thus compatible with pT7-7 derived constructs. E. coli B strains like BL21 lack the *lon* protease and the *ompT* outer membrane protease. These deficiencies can help to stabilise the expression and accumulation of foreign proteins in E. coli. Assays of crude extracts of E. coli BL21(DE3) containing each of the three expression constructs described above indicated that the highest levels of expression of luciferase were obtained from cells containing the construct pPW304 (see Table 2).

The mutant proteins of the invention provide advantages other than thermostability. It has been found that the mutation of the amino acid at position Photinus 354/Luciola 356 provided a change in wavelength of light emitted on oxidation of luciferin dependent upon the amino acid or analogue with which the glutamate is substituted. Thus the invention also provides luciferases for use as specific binding agent labels or reporter genes which report back identity as a specific wavelength of light when the luciferin oxidation using their protein products; such property gives utility to such mutations as glycine, proline and aspartate. A further advantage of the proteins of the invention, deriving from their increased thermostability, is the ability to produce them at higher temperature, eg. at 37°C or above, with correspondingly increased yield, as is exemplified below.

The proteins, DNA, vectors and cells of the invention will now be described by way of illustration only by reference to the following non-limiting Examples, Figures, Tables and Sequence listing. Further proteins, conjugates of proteins, DNA, vectors and cells, and assays and test kits incorporating any of the above will occur to those skilled in the art in the light of these.

FIGURES

Figure 1: shows a restriction map of plasmid pPW204 derived from pKK223-3 by insertion of a *luc* gene as described in the Examples below.

Figure 2: shows a restriction map of plasmid pPW116 derived from pDR540 by insertion of a *luc* gene as described in the Examples below.

Figure 3: shows a restriction map of plasmid pPW304 derived from pT7-7 by insertion of a *luc* gene as described in the Examples below.

Figure 4: shows a restriction map of plasmid pPW601a derived from pDR540 and BamH1/Sst1 fragment from pGEM-*luc* with the Xho site removed.

Figure 5: shows a graph of heat inactivation of recombinant and wild type Photinus luciferases (Sigma) incubated at a given temperature for 20 minute periods as described in the Examples below.

Figure 6: shows a graph of luciferase activity in crude extracts of E. coli BL21(DE3)pPW304 during growth at different temperatures.

Figure 7: shows a graph of heat inactivation of activity of luciferases derived from pPW304 and pPW304M-1 (plasmid of the invention encoding such that lysine replaces glutamate 354).

Figure 8: shows a graph of time dependent inactivation of Sigma wild type, and pPW304 and pPW304M-1 recombinant luciferases at 37°C.

Figure 9: shows a restriction map of pT7-7 after Tabor.

Figure 10: shows a graph illustrating heat inactivation in Promega lysis buffer at 40°C of activity of crude cell extracts of luciferase expressing E. coli of the invention expressing luciferases having

substitutions of alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, glutamine, histidine, asparagine, methionine, arginine, lysine, serine, threonine and cysteine respectively for the wild type glutamate at position 354.

Figure 11: shows a graph illustrating heat inactivation of activity of purified double mutant luciferase having the E354K Lysine and the A 215L Leucine changes at 47°C in phosphate buffer as compared to the single mutants A215L and E354K.

Figure 12: shows a graph of % initial activity of the Lysine E354K mutant, recombinant wild-type and native firefly luciferases remaining against time at 37°C in pH7.75 HEPES buffer with 0.02% azide.

Figure 13: shows a graph of luciferase expression at 37°C for recombinant wild-type, E354K single and E354K+A215L double mutants with increase in optical density as a measure of culture cell density plotted against luciferase activity.

Figure 14: shows a graph of % initial activity against time of 10ng/ml of each of the A215L and E354K single, A215L+E354K double, recombinant and Sigma wild-type luciferases over 5 hours in HEPES, pH7.75 containing 1%BSA and 0.02% azide at 37°C.

Figure 15: shows a graph of % initial activity against time of 10ng/ml of each of the A215L and E354K single, A215L+E354K double, recombinant and Sigma wild-type luciferase over 5 hours in HEPES pH7.75 containing 1%BSA, 0.02% azide, 2mM EDTA and 2mM DTT at 37°C.

SEQUENCE LISTING:
The sequence listing provided at the end of this specification describes DNA and amino acid sequences as follows:

SEQ ID NO 1: shows the DNA sequence of a DNA encoding for luciferase

of the invention wherein the Photinus pyralis wild-type codon at 1063 to 1065 is mutated; for lysine the base at 1063 is mutated to an A.

SEQ ID No 2: shows the amino acid sequence of a protein of the invention wherein the Photinus pyralis wild-type amino acid 354 glutamate has been changed to another amino acid.

SEQ ID No 3: shows the sequence of the oligonucleotide used for the SDM mutation of pPW601 to give a lysine instead of glutamate at position 354 in Example 2.

SEQ ID No 4: shows the sequence of the oligonucleotide used for the SDM mutation of pPW601 to give leucine at position 215 in Example 5.

SEQ ID No 5: shows the amino acid sequence of a protein of the invention wherein the Photinus pyralis wild-type amino acid 354 glutamate has been changed to any other amino acid and the 215 amino acid changed to a leucine.

EXAMPLES

EXAMPLE 1: Production of plasmids containing DNA of the invention.

Plasmids pKK223-3 and pDR540 were obtained from Boehringer Mannheim; pDR540 is also available from Pharmacia.

Plasmid pT7-7 (see Current protocols in Molecular Biology Vol II Section 16.2.1) was obtained from Stan Tabor, Dept of Biol Chem, Harvard Medical School, Boston, Mass 02115 and (as shown in Figure 8) contains T7 RNA polymerase promoter ϕ10 and the translation start site for the T7 gene 10 protein (T7 bp 22857 to 22972) inserted between the PvuII and ClaI sites of pT7-5. Unique restriction sites for creation of fusion proteins (after filling in 5' ends) are Frame 0: EcoR1; Frame 1: NdcI, SmaI, ClaI; Frame 2: BamHI, SalI, HindIII. SacI site of the original polylinker is removed by deletion and an additional XbaI site is provided upstream of the start codon.

Firefly luciferase (prepared from a crystalline suspension, Cat No L9009), coenzyme A and ATP were obtained from Sigma Chemical Co. Beetle luciferin potassium salt was obtained from Promega. Cell extracts were prepared as described in the Promega technical bulletin No 101. Aliquots of E. coli cultures were lysed in cell culture lysis reagent (25mM Tris-phosphate, pH7.8, 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X-100, 2.5mg/ml BSA, 1.25mg/ml lysozyme) for 10 minutes at room temperature and then stored on ice prior to assay.

Luciferase activity of cell lines was assayed by monitoring bioluminescence emitted by colonies by transferring these to nylon filters (Hybond N, Amersham) and then soaking the filters with 0.5mM luciferin in 100mM sodium citrate buffer pH5.0 (Wood & DeLuca, (1987) Anal Biochem 161 p501-507). Luciferase assays in vitro were performed at 25°C using 125µl of assay buffer (20mM Tricine, 1mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 0.27mM coenzyme A, 0.47mM luciferin, 0.53mM ATP and 1 to 2µl of sample). The final pH of the assay cocktail was 7.8 and light measurements were made with a BioOrbit 1250 luminometer.

For production of non-specific chemical mutations of DNA, plasmids containing *luc* genes were treated according to the method of Kironde et al (1989) Biochem. J. 259, p421-426 using 0.8M hydroxylamine, 1mM EDTA in 0.1mM sodium phosphate pH6.0 for 2 hours at 65°C. The mutagenised plasmid was desalted on a G60 DNA grade Nick column (Pharmacia) followed by transformation into E. coli BL21(DE3).

Heat inactivation studies were carried out by incubating crude cell extracts having luciferase activity at various temperatures for 20 minutes and measuring remaining activities. In studies with the purified luciferase obtained from Sigma the enzyme was diluted in Promega lysis buffer prior to inactivation. For time dependent studies Eppendorf tubes containing 50µl of crude cell extract or Sigma luciferase in lysis buffer were incubated at 37°C. At various times a

tube was removed and cooled on ice prior to assay. The remaining activity was expressed as per cent of original activity.

Relative levels of expression of luciferase from each of the constructs pPW204, pPW116 and pPW304 are 0.1:0.5:1.0 from E. coli BL21(DE3). Cells were grown in LB at 37°C to an OD 600 of 0.3 then induced with IPTG and growth allowed to continue for 4 hours after which crude extract was prepared and luciferase activity measured.

TABLE 1: Ribosome binding sites (underlined) and start codons in the expression constructs used in Example 1.

pPW304 AAGGAGATATACAT ATG* CGT AGA ATT CAA ATG
pPW116 AGGAAACAGGATCCA ATG*
pPW204 AGGAAACAGCAA ATG*

The site directed mutagenesis required to convert the glutamate to an alternative amino acid was carried out using the following protocol. Because the glutamate to lysine mutation lies within a unique AvaI restriction site, and thus destroys it, it is possible to use a single oligonucleotide as the mutagenic and selection oligonucleotide.

Site Directed Mutagenesis Protocol:
Plasmid selected is denatured and annealed with a selection/mutagenic oligonucleotide for lysine: 5'-CATCCCCCTTGGGTGTAATCAG-3' with the underlined T being the mismatch. The mutant DNA strand is synthesised and ligated and the whole primary restriction digested with AvaI.

Transformation into cells, here E. coli BMH 71-18 mut S cells, was carried out using a Bio-Rad Gene Pulser version 2-89. Harvested cells and purified mixed plasmid pool containing mutated and parental plasmids were provided and secondary restriction digest with AvaI was carried out before transformation into E. coli JM109 cells. These cells were plated on selective media (LB agar + 50 µg/ml ampicillin) and clones screened by purifying their plasmid DNA and analysing for

the loss of the AvaI restriction site. Plasmid DNA was purified in each case using the alkaline lysis method of Birnboim and Doly (1979) Nucleic Acids Research 7, p1513. Precise protocols were as described in the Transformer^{RTM} Site -Directed Mutagenesis Kit (Version 2) sold by Clontech Laboratories Inc (US) catalog No K1600-1.

The restriction map for pPW601a, a variant of pPW116 derived from Pharmacia pDR540 and BamH1/Sst1 fragment from pGEM-*luc* with the Xho site destroyed is shown as Figure 4. Site directed mutagenesis was carried out as described above and in the Clontech instructions such as to convert the wild-type Photinus *luc* gene inserted therein into a sequence as shown in SEQ ID No 1 wherein 1063-1065 is AAG, with expressed protein of amino acid sequence modified at position 354 as shown in SEQ ID No 2 to Lysine.

EXAMPLE 2: Heat stability of luciferases:

The heat stability of various luciferases expressed by unmodified and modified (ie. of the invention) *luc* genes in vectors in E. coli produced as described above was determined and results are shown in Figures 5 to 8.

A comparison of $t^{1/2}$ (half-life) of the activity of 50µg/ml luciferase at 43.5°C in 50mM potassium phosphate buffer pH7.8, 1mM EDTA, 0.2%(w/v) BSA, 1mM DTT and 10% ammonium sulphate shows 50% activity remaining to be reached at times as follows:

Sigma wildtype luciferase:	$t^{1/2}$ reached in approximately	1.5 minutes
pPW601 (354=glutamate):	$t^{1/2}$ reached " " "	5 "
pPW601aK (354=lysine):	$t^{1/2}$ reached " " "	30 "

Thus clearly from the aforesaid figures it can be seen that replacing the 354 glutamate with lysine increases heat stability of luciferase at least up to 43.5°C.

EXAMPLE 3: Heat stability of luciferase:

The heat stability of a number of luciferases expressed by SDM modified *luc* genes corresponding to other position 354 mutations of the invention in vectors in E. coli produced by methods analogous to that as described in Example 1 was determined and results are graphically shown in Figure 10.

A comparison of $t^{1/2}$ at 40°C in Promega lysis buffer was carried out and results obtained in $t^{1/2}$ in minutes as:

pPW601aK	(354=lysine)	$t^{1/2}$ reached in approximately	13 minutes
pPW601aR	('' =arginine)	$t^{1/2}$ reached '' '' ''	13 ''
pPW601aL	('' =leucine)	$t^{1/2}$ reached '' '' ''	10 ''
pPW601aI	('' =isoleucine)	$t^{1/2}$ reached '' '' ''	10 ''
pPW601aN	('' =asparagine)	$t^{1/2}$ reached '' '' ''	10 ''
pPW601aV	('' =valine)	$t^{1/2}$ reached '' '' ''	9 minutes
pPW601aW	('' =tryptophan)	$t^{1/2}$ reached '' '' ''	8 ''
pPW601aA	(354=alanine)	$t^{1/2}$ reached '' '' ''	6.5''
pPW601aY	('' =tyrosine)	$t^{1/2}$ reached '' '' ''	6.5''
pPW601aM	('' =methionine)	$t^{1/2}$ reached '' '' ''	5.5''
pPW601aF	('' =phenylalanine)	$t^{1/2}$ reached '' '' ''	5 ''
pPW601aH	('' =histidine)	$t^{1/2}$ reached '' '' ''	5 ''
pPW601aT	('' =threonine)	$t^{1/2}$ reached '' '' ''	4.5''
pPW601aQ	('' =glutamine)	$t^{1/2}$ reached '' '' ''	4.5''
pPW601aC	('' =cysteine)	$t^{1/2}$ reached '' '' ''	4 ''
pPW601aS	('' =serine)	$t^{1/2}$ reached '' '' ''	3.5''
pPW601aE	('' =glutamic acid)	$t^{1/2}$ reached '' '' ''	1 ''
pPW601aD	('' =aspartic acid)	$t^{1/2}$ reached '' '' ''	1 ''
pPW601aP	('' =proline)	$t^{1/2}$ reached '' '' ''	1 ''
pPW601aG	('' =glycine)	$t^{1/2}$ reached '' '' ''	<1 ''

EXAMPLE 4: Stability of Luciferases at 37°C and room temperature.

Luciferases of pPW601K lysine mutation (86ng/ml), recombinant wild type (550ng/ml) and native type (Sigma) (62.5 ng/ml) were incubated for 4 hours at 37°C in 1% BSA, pH7.75 HEPES buffer with 0.02% azide as

preservative. To measure remaining activity 1ng luciferase was added to D-luciferin substrate and luminescent counts per minute recorded.

Results are shown below in terms of remaining activity after incubation for 2 hours at 37°C and after 10 days at room temperature.

After 2 hours at 37°C:	
E354K mutant luciferase	70% remaining activity
Recombinant Wild Type luciferase	12% " " "
Sigma Native luciferase	18% " " "
After 10 days at Room temperature:	
E354K mutant luciferase	85% " " "
Recombinant Wild Type luciferase	59% " " "
Sigma Native luciferase	71% " " "

EXAMPLE 5: Preparation and stability of 354K:215L double mutant.

The double mutant 354 Lysine:215 Leucine of pPW601a *Photinus pyralis* luciferase was prepared by taking pPW601aE354K as described in Example 1 and mutating it using the oligonucleotide of SEQ ID No 4 5'-GAATCTGACGCAGAGAGTTCTATGCGG-3', wherein the underlined bases represent the mismatches that cause the mutation. This mutation was confirmed by DNA sequencing and measurement of the thermostability of the resultant luciferase as expressed in *E.coli* by a method analogous to that as described in Example 1 was carried out as in Examples 2 to 4 using pH7.8 phosphate buffer containing 1mM EDTA, 0.2% (w/v) BSA, 1mM DTT and 10% ammonium sulphate as heat inactivation medium.

At 43.5°C in the phosphate buffer there was less than 5% loss of activity over 32 minutes, while at 47°C $t^{1/2}$ was approximately 38 minutes. At 50°C the double mutant retains 15% activity after 16 minutes incubation. Results for this inactivation test are shown graphically in Figure 12.

EXAMPLE 6: Purification of Luciferases.

E. coli JM109 cells expressing the recombinant wild-type or mutant luciferases were grown at 30°C in Luria Broth (LB) containing 50µg/ml ampicillin and induced with IPTG (1mM) during early log phase. Cells were harvested in mid stationary phase and resuspended in 50mM Tris-HCl pH8.0 containing 50mM KCl, 1mM dithiothreitol, 1.2mM phenylmethylsulphonylfluoride (PMSF) and 1mM EDTA (Buffer A). Cells were broken by disruption in an MSE soniprep 150 sonicator (amplitude 14µ) and the cell lysate centrifuged at 30000 x g for 30 minutes. The supernatant of the crude extract was then subjected to fractionation with ammonium sulphate with the fraction precipitated between 35% and 55% saturation being found to contain luciferase activity and being dissolved in Buffer A.

The extract was desalted using a Pharmacia PD10 column equilibrated in 50mM Tris-HCl pH8.0 containing 0.5mM DTT (Buffer B) and the desalted extract applied to a Pharmacia Mono Q anion-exchange column and eluted with a linear gradient of 0 to 500mM NaCl in Buffer B at a flow rate of 4ml/minute in 2 ml fractions. The peak fraction of luciferase activity was collected and dialysed against 25mM sodium phosphate buffer, pH7.5, containing 0.5mM DTT and 12% (v/v) glycerol for long term storage.

EXAMPLE 7: Heat inactivation of purified luciferases.

Eppendorf tubes containing cell free extracts of luciferase were prepared as described in Example 6. Purified preparations of luciferase (50µg/ml) were incubated in thermostability buffer comprising 50mM potassium phosphate buffer pH7.8 containing 10% saturated ammonium sulphate, 1mM dithiothreitol and 0.2% bovine serum albumin (BSA). At set times a tube was removed and cooled in an ice/water bath prior to assay with remaining assayed activity being calculated as a percentage of the initial activity.

Arrhenius plots for purified recombinant wild-type and thermostable

luciferases were constructed by measuring the half-life for inactivation in thermostability buffer over a range of temperatures from 42°C to 50°C. The natural log of t1/2 in minutes was then plotted against 1/K. For an equivalent rate of inactivation the E354K mutation increases thermostability by 2°C at temperatures in this range as compared with an increase of 5°C with the A215L mutation and 6°C for the double mutant E354K+A215L; the latter showing the additive nature of the double mutation.

EXAMPLE 8: Increased expression of mutant luciferases as compared to wild-type recombinant luciferase in E.coli.

Expression of luciferase in E. coli JM109 cells was monitored during growth in liquid culture at 37°C. Cells expressing the thermostable mutants being found to accumulate more active luciferase during growth than cells expressing the recombinant wild-type enzyme. Figure 13 shows this effect graphically in plotting luciferase activity with increasing optical density at 600nm for cultures of recombinant wild-type, E354K+A215L double mutant and E354K. It can be seen that the increased thermostability of the single and double mutant allows increased production of luciferase at the 37°C culture temperature.

EXAMPLE 9: Effect of buffer on stability of mutant luciferases at 37°C.

10ng/ml solutions of each of the A215L, E354K, E354+A215L, recombinant wild-type and sigma luciferases were prepared in HEPES pH7.75 buffer with 1% BSA and 0.02% azide and thermostability at 37°C compared to that of the same compositions with addition of 2mM EDTA and 2mMDTT. Results are shown graphically in Figures 14 and 15 indicating that the relative stability of A215L and E354K varies with buffer at 37°C.

EXAMPLE 10:Effect of amino acid substitution on wavelength of light emitted in oxidation of D-luciferin.

The wavelength of light emitted on oxidation of D-luciferin with the various luciferases of the invention set out in Example 3 was measured and found to vary with the amino acid mutation. The

wavelength of light emitted varied 5nm between recombinant wild-type (E354) and E354K, and about 15nm between E354K and E354I.

Wild-type recombinant E. coli organisms give a yellow green luminescence in the presence of D-luciferin. Colours emitted by the respective mutant E. coli when provided with D-luciferin were as follows:

E354G	yellow-green
E354N	yellow-green
E354A	green
E354V	orange-red
E354M	orange-red
E354F	yellow-green
E354L	yellow
E354Y	yellow-green
E354S	yellow-green
E354C	yellow-green
E354K	yellow
E354Q	yellow-green
E354W	yellow-green
E354T	yellow-green
E354P	orange
E354R	yellow-orange
E354H	yellow-green
E354N	yellow
E354I	red

SEQUENCE LISTING

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(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SP4 0JQ

(ii) TITLE OF INVENTION: LUCIFERASES
(iii) NUMBER OF SEQUENCES: 5
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release @1.0, Version @1.25 (EPO)
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9405750.2
(B) FILING DATE: 23-MAR-1994
(vi) APPLICATION NUMBER: GB 9501170.6
(B) FILING DATE: 20-JAN-1995
(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1722 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 4..1653
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CAAATGGAAG ACGCCAAAAA CATAAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT 60

GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA 120

ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG 180

TCCGTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC 240

GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA 300

GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC 360

ATTTCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG 420

CAAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG 480

GGATTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC 540

GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT 600

GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC 660

TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT 720

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA 780

TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT 840

TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT 900

CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT 960

TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA 1020

TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCNNNGGGGA TGATAAACCG 1080

GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG 1140

AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC 1200

GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT 1260

TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG 1320

TCTTTAATTA AATACAAAGG ATATCAGGTG GCCCCCGCTG AATTGGAATC GATATTGTTA 1380

CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA 1440

CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG 1500

GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG 1560

GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC 1620

ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC 1680

GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA 1722

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis
(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 354

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35 40 45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Xaa Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu 465
470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis
(ix) FEATURE:
(A) NAME/KEY: misc-.difference
(B) LOCATION: replace(10, "")

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATCCCCCTT GGGTGTAATC AG 22

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(16..17, "")
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAATCTGACG CAGAGAGTTC TATGCGG 27

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis
(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 354
(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 215

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35 40 45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Leu Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Xaa Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550

CLAIMS.

1. A protein having luciferase activity and having over 60% homology of amino acid sequence to luciferase from Photinus pyralis, Luciola mingrelica, Luciola cruciata or Luciola lateralis characterised in that the amino acid residue corresponding to residue 354 of Photinus pyralis luciferase or residue 356 of Luciola mingrelica, Luciola cruciata and Luciola lateralis luciferase is an amino acid other than glutamate.

2. A protein as claimed in claim 1 characterised in that it comprises an amino acid sequence XGDDKPGA wherein X is the amino acid residue other than glutamate.

3. A protein as claimed in claim 2 characterised in that it comprises an amino acid sequence TPXGDDKPGA wherein X is the amino acid residue other than glutamate.

4. A protein as claimed in claim 1, 2 or 3 characterised in that the amino acid X is not glycine, proline or aspartic acid.

5. A protein as claimed in claim 1, 2 or 3 characterised in that the amino acid X is one of tryptophan, valine, leucine, isoleucine and asparagine or an analogue or modification of any of these.

6. A protein as claimed in claim 1, 2 or 3 characterised in that the amino acid X is one of lysine and arginine or an analogue or modification of these.

7. A protein comprising an amino acid sequence as described in SEQ ID No 2 wherein Xaa is an amino acid as listed in any one of claims 5 or 6 or an analogue or modification thereof.

8. A DNA encoding for a protein as claimed in any one of claims 1 to 7.

9. A DNA as claimed in claim 8 comprising a nucleotide sequence as described in SEQ ID No 1 wherein the three bases N at 1063 to 1065 form a codon encoding for an amino acid other than glutamate.

10. A DNA as claimed in claim 9 wherein the codon encodes for an amino acid, analogue or modification as listed in claim 5 or 6.

11. A vector comprising a *luc* gene encoding for a protein as claimed in any one of claims 1 to 7.

12. A vector as claimed in claim 11 obtainable by treating a vector containing a wildtype or recombinant *luc* gene by site directed mutagenesis to change the codon responsible for encoding for the glutamate at position 354 of Photalis pyralis luciferase or the glutamate at position 356 of Luciola mingrelica, Luciola cruciata or Luciola lateralis luciferase to an alternative amino acid, analogue or modification thereof.

13. A vector as claimed in claim 12 wherein the alternative amino acid is an amino acid, analogue or modification as listed in any one of claims 5 or 6.

14. A vector as claimed in any one of claims 9 to 13 selected from pKK223-3, pDR540 and pT7-7 into which a *luc* gene has been ligated.

15. A cell capable of expressing a protein as claimed in any one of claims 1 to 7 comprising DNA or a vector as claimed in any one of claims 8 to 14.

16. A cell as claimed in claim 15 being an E. coli, S. cerevisiae or an insect cell.

17. A test kit for performance of an assay through measurement of ATP characterised in that the kit comprises a protein as claimed in any one of claims 1 to 7 contained within a luminescent reagent.

18. An assay method wherein ATP is measured using luciferin and luciferase to generate light the quantity of which is related to the amount of ATP characterised in that the luciferase is a protein as claimed in any one of claims 1 to 7.

19. An assay method as claimed in claim 18 wherein the assay is carried out at a temperature of from 30°C to 70°C.

20. An assay method as claimed in claim 18 wherein the assay is carried out at a temperature of from 37°C to 60°C

21. An assay method as claimed in claim 18 wherein the assay is carried out at a temperature of from 40°C to 50°C.

22. A luciferase preparation that retains 85% or more of its luciferase activity when stored at room temperature for 10 days at room temperature in the absence of thermostabilising agent.

23. Use of a luciferase as claimed in any one of claims 1 to 7 or 22 as a label for a specific binding reagent.

24. A test kit characterised in that it comprises a specific binding reagent labelled with a luciferase as claimed in any one of claims 1 to 7.

25. Use of a luciferase encoding DNA or vector as claimed in any one of claims 8 to 14 for the purpose of reporting the identity of a cell or DNA.

Fig.1.

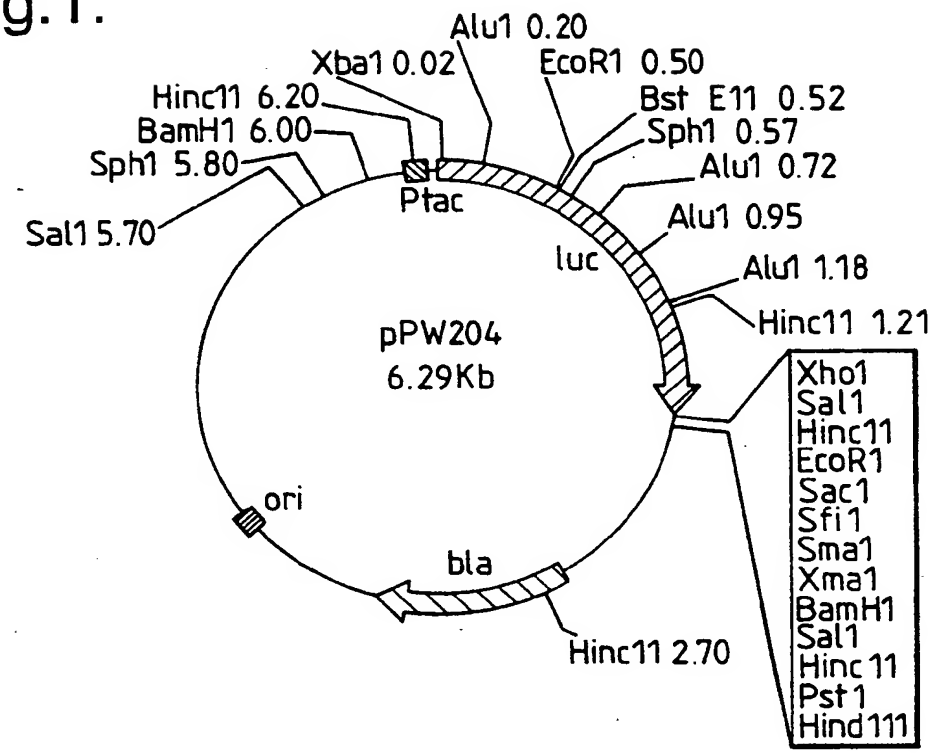


Fig.2.

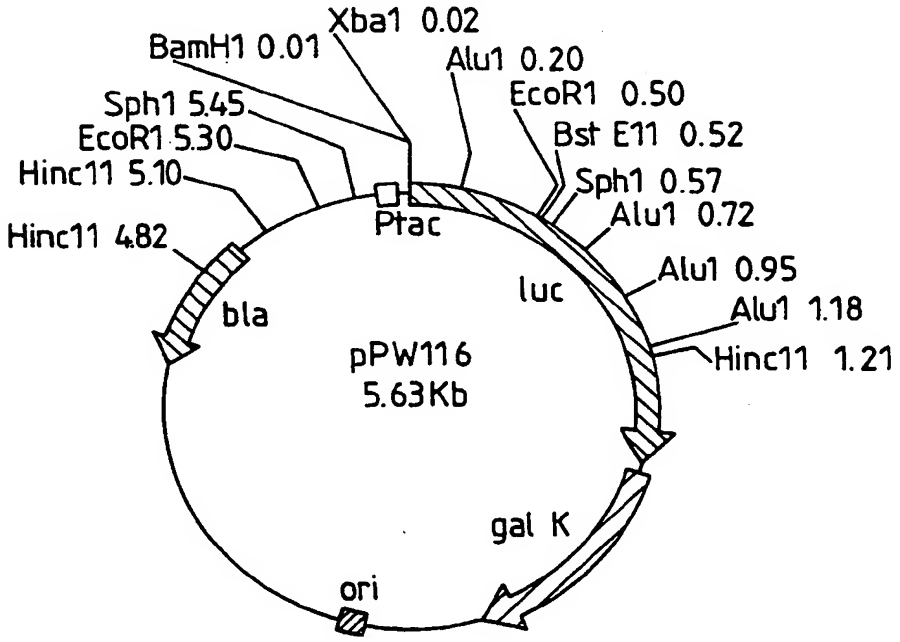


Fig.3.

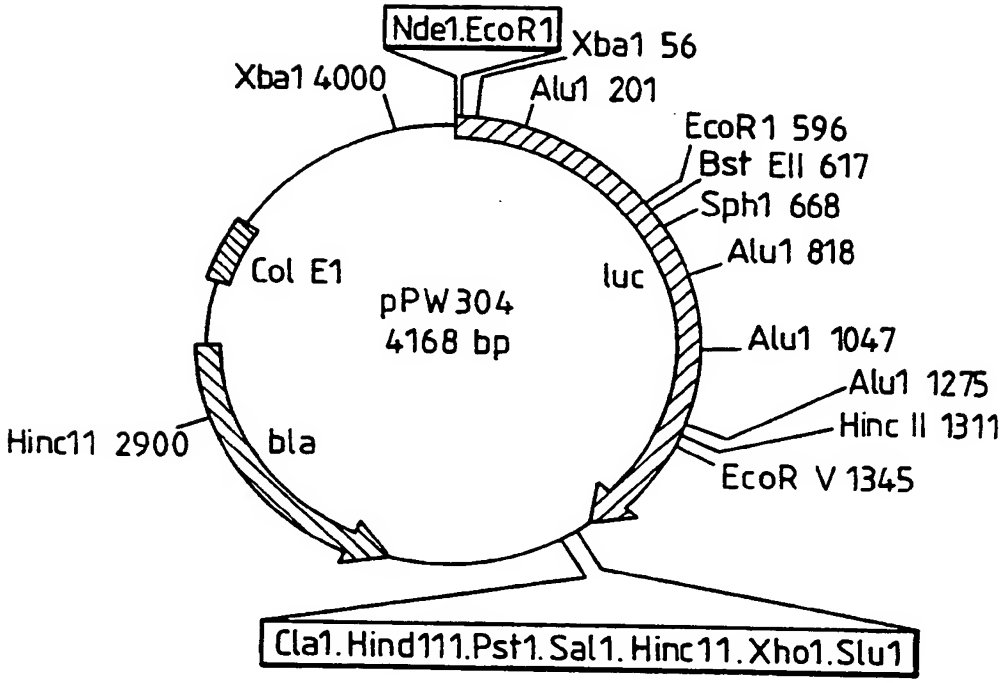
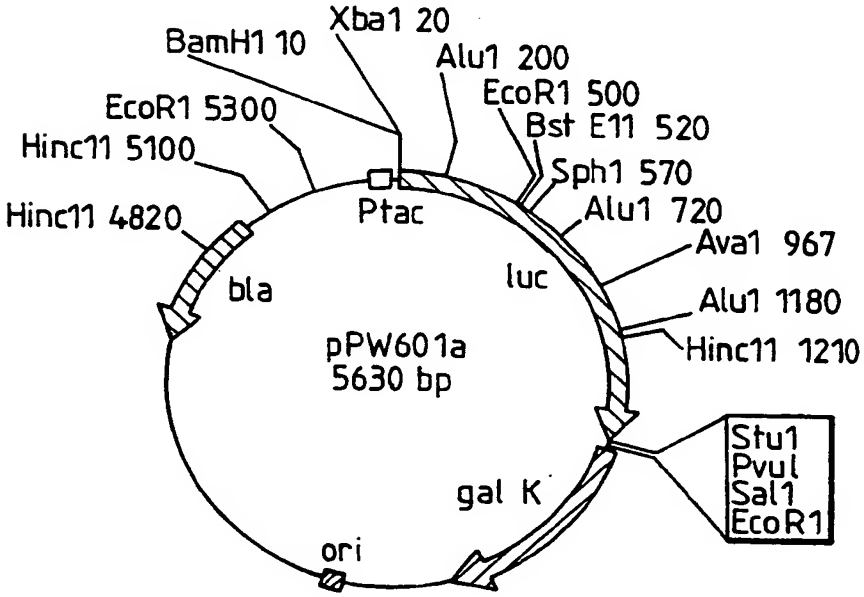


Fig.4.



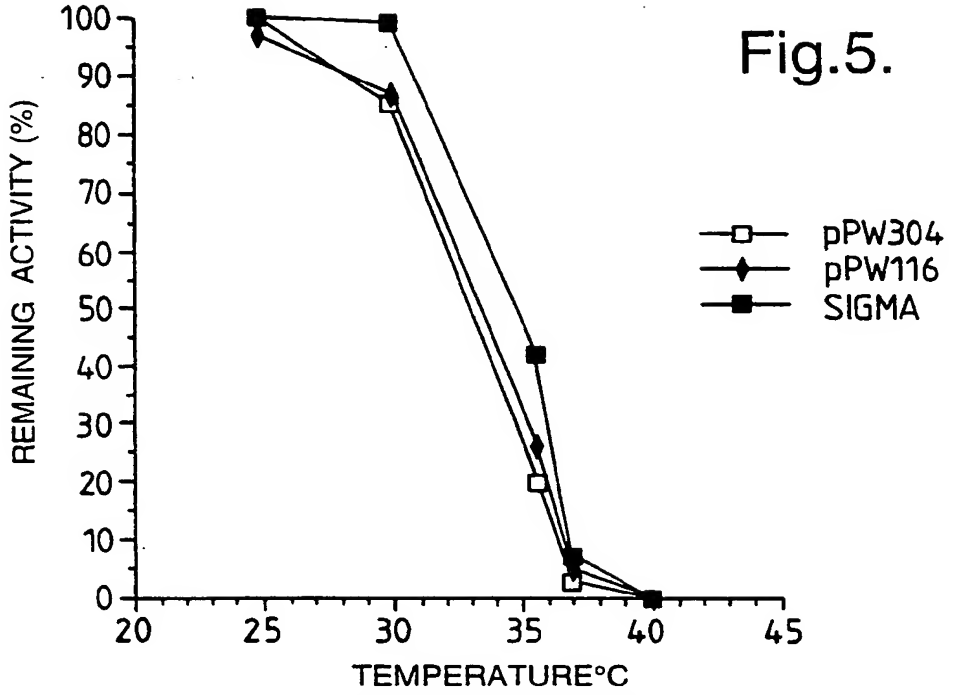
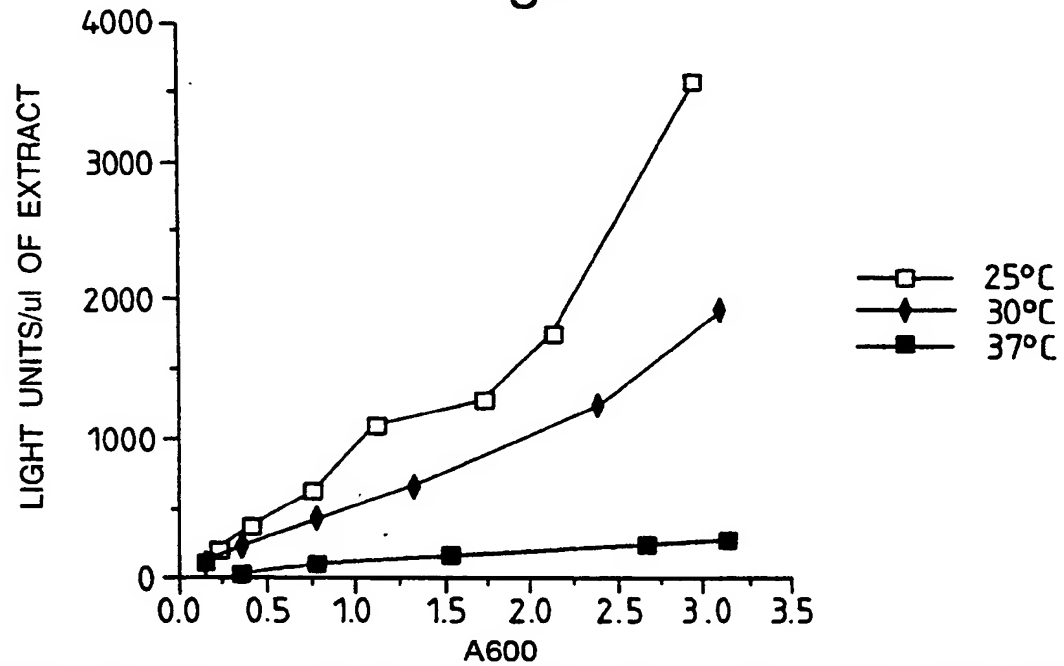


Fig.5.

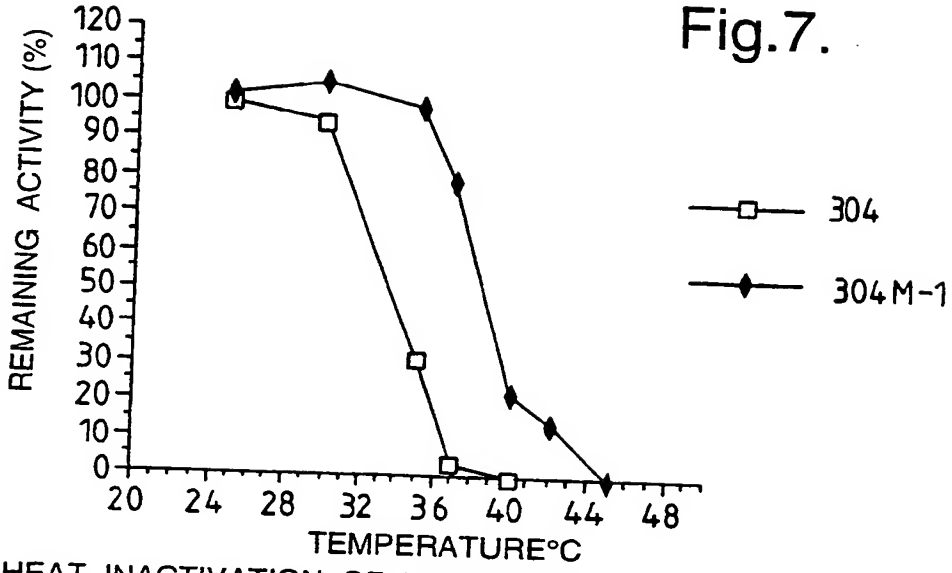
HEAT INACTIVATION OF RECOMBINANT AND WILD-TYPE (SIGMA) LUCIFERASES. ENZYMES WERE INCUBATED FOR 20min AS DESCRIBED IN METHODS.

Fig.6.



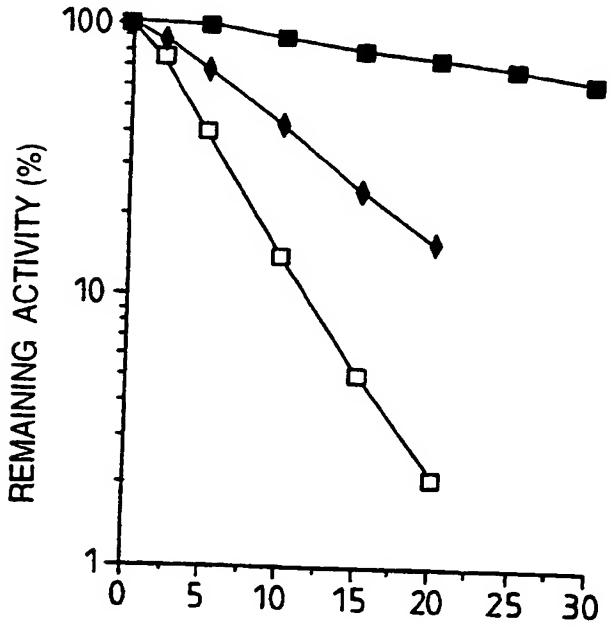
LUCIFERASE ACTIVITY IN CRUDE EXTRACTS OF E. COLI BL21 (DE3) pPW304 DURING GROWTH AT DIFFERENT TEMPERATURES

Fig.7.



HEAT INACTIVATION OF LUCIFERASE 304 AND 304M-1.
ENZYMES WERE INCUBATED FOR 20min AS DESCRIBED IN METHODS.

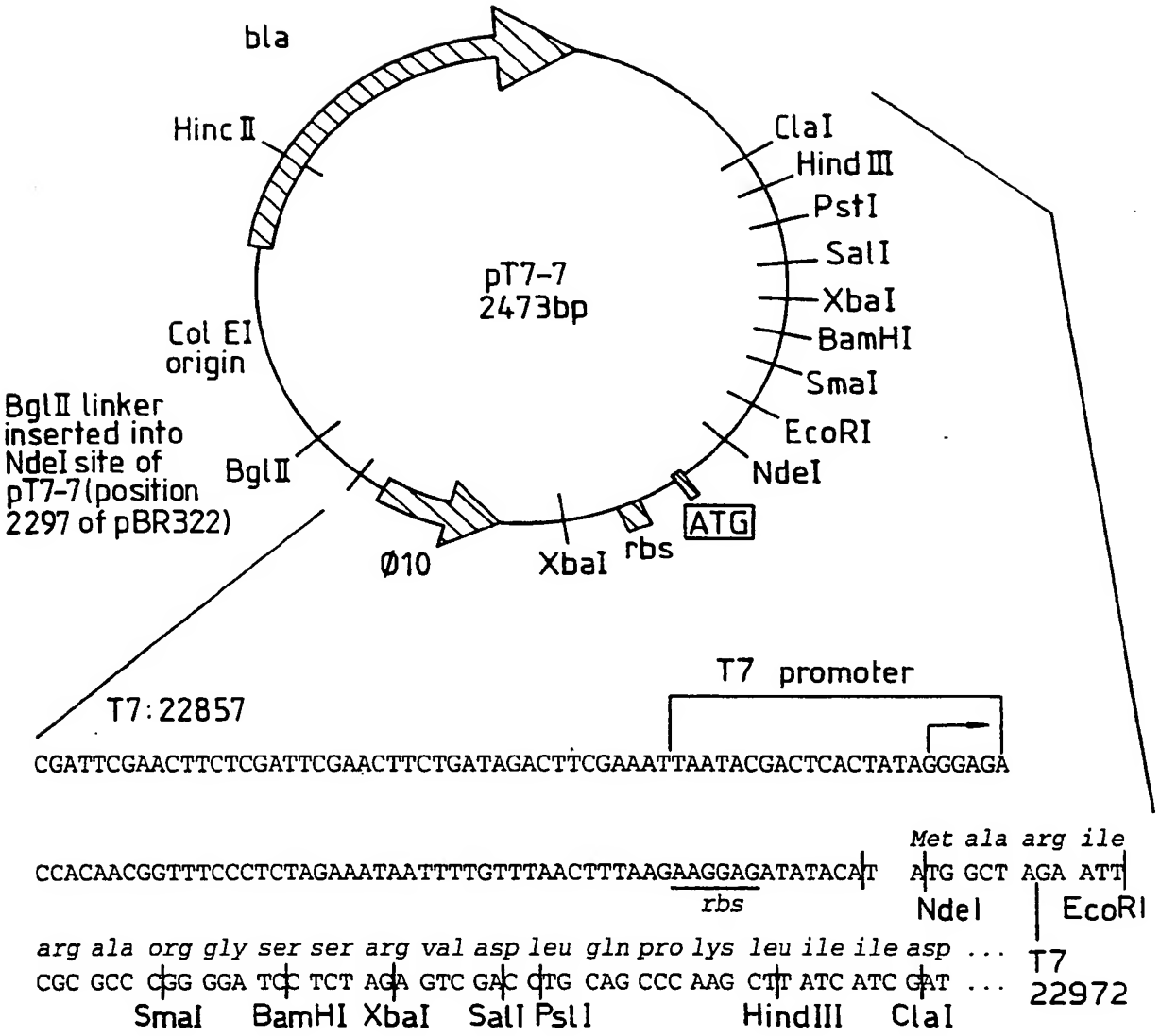
Fig.8.



	t1/2(min)
304	3.75
SIGMA	7.5
304M-1	50.0

TIME DEPENDENT INACTIVATION OF LUCIFERASES AT 37°C

Fig.9.



INACTIVATION OF E354X MUTANTS IN PROMEGA LYSIS BUFFER (40°C)

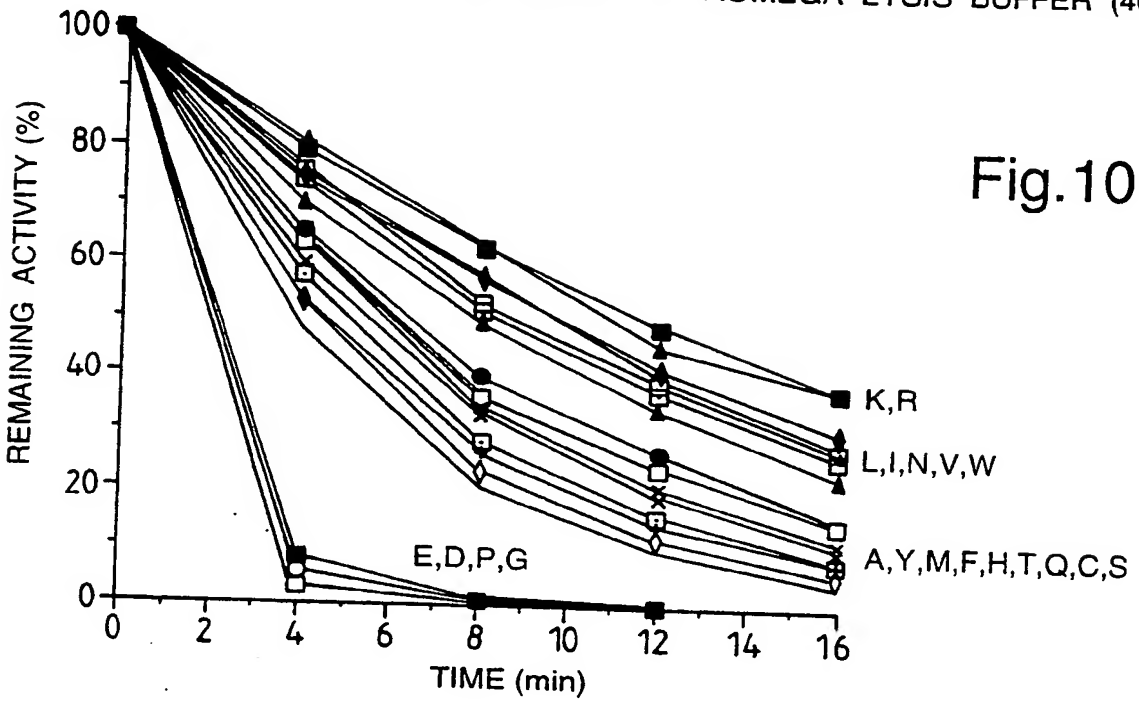


Fig.10.

HEAT INACTIVATION AT 47°C

Fig.11.

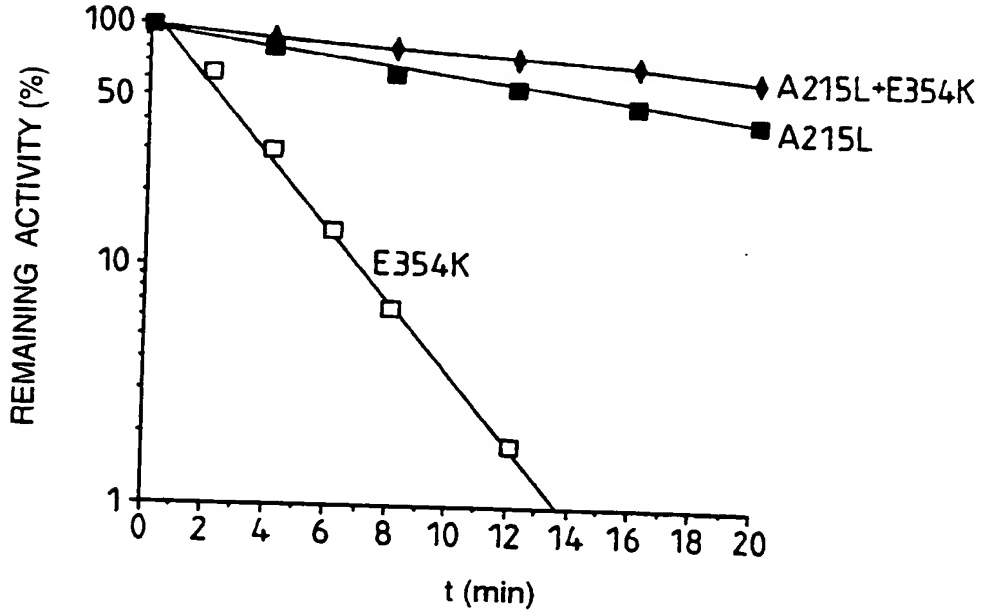


Fig.12.

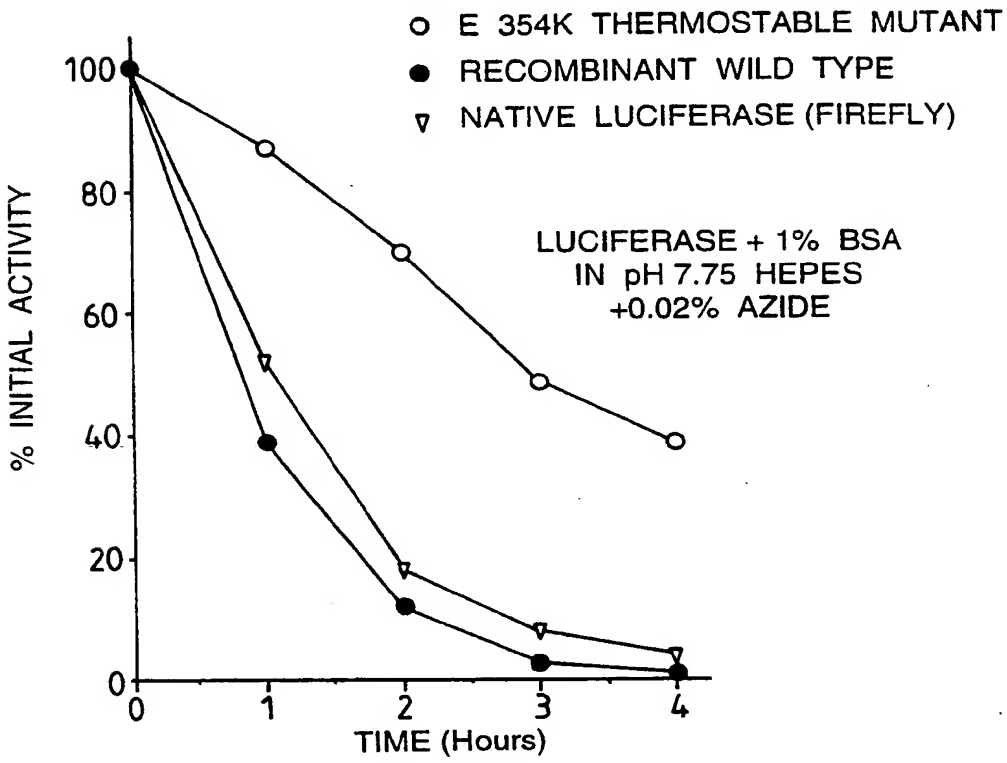


Fig.13.

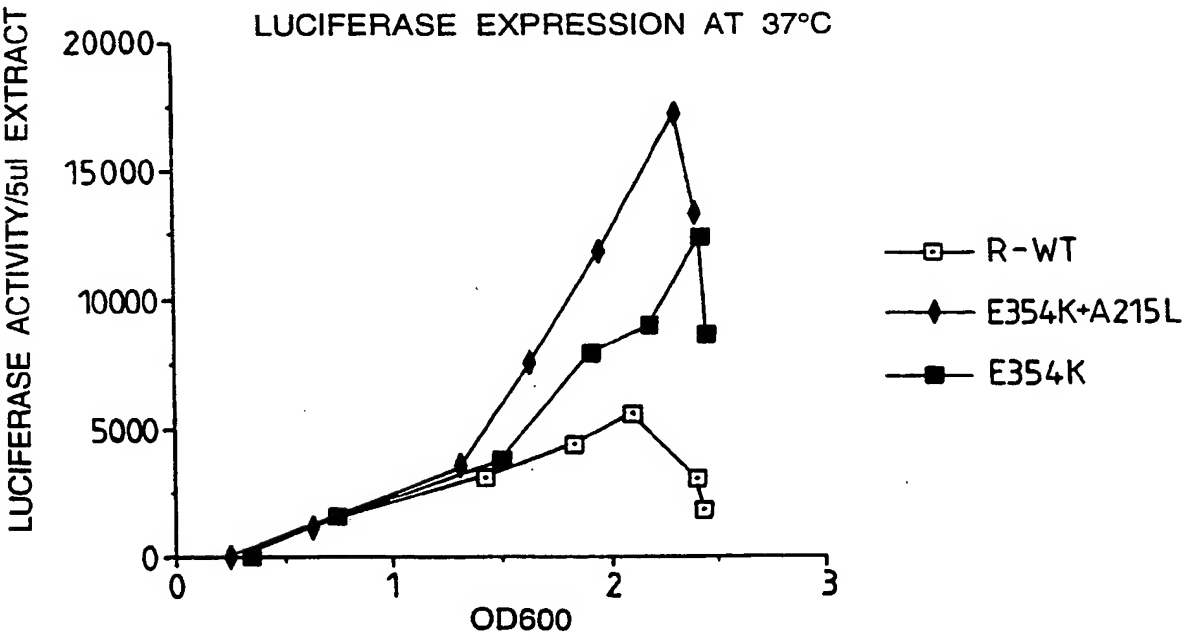
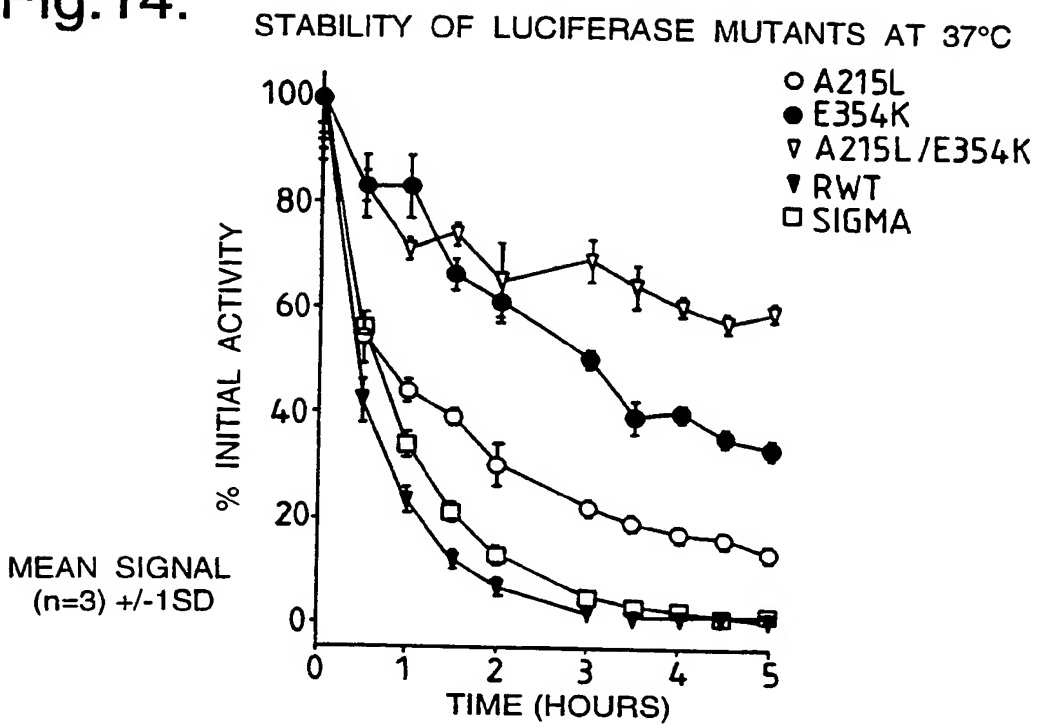
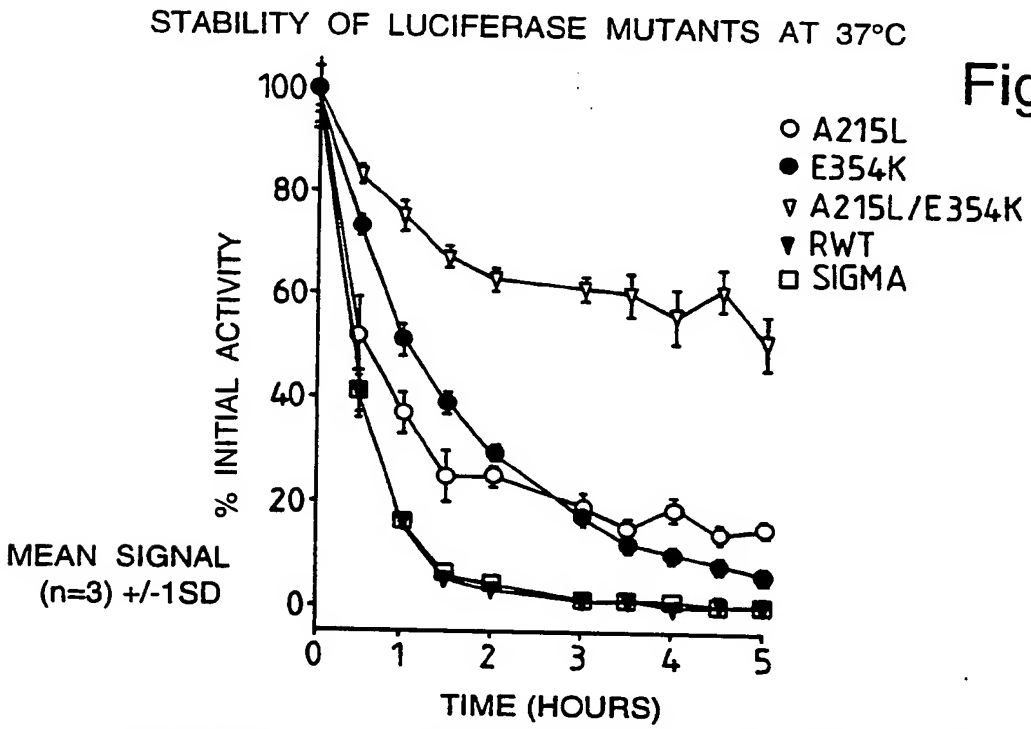


Fig.14.



ENZYMES AT 10ng/ml IN HEPES, pH 7.75 CONTAINING
1% BSA AND 0.02% AZIDE

Fig.15.



ENZYMES AT 10ng/ml IN HEPES, pH 7.75 CONTAINING
1% BSA AND 0.02% AZIDE, 2mM EDTA AND 2mM DTT

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 95/00629

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 G01N33/50 C12Q1/68 C12N1/19
C12N1/21 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Database, Accession No.: X65316, Identification: CVPGEMLUC, Promega cloning vector pGEM-luc see bp 693-695 ---	1-5, 7-13,15, 16,22
X	EP,A,0 524 448 (KIKKOMAN CORPORATION) 27 January 1993 cited in the application see the whole document -----	22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1995	01.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Espen, J

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INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter ...al Applicat... No
PCT/GB 95/00629

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0524448	27-01-93	US-A-	5229285	20-07-93
		JP-A-	5244942	24-09-93

Form PCT/ISA/210 (patent family annex) (July 1992)